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THE INACTIVATION OF TRYPSIN. I.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 15, 1921.)

In most of the early work of the kinetics of enzyme action it was tacitly assumed that enzyme reactions obeyed the law of mass action and that they represented a special case of homogeneous catalysis. As the individual enzymes were studied more carefully, however, it became apparent that few, if any, obeyed the law of mass action in its simplest form. Several authors have, therefore, taken the view that enzyme reactions should be considered as occurring in heterogeneous systems, and that owing to their colloidal nature they do not conform to the law of mass action. On the other hand, Tammann, Michaelis, Arrhenius,[1] Euler,[2] Taylor,[3] and others consider that in general the law of mass action does hold and that the apparent divergencies are due to secondary reactions. The author has found that the peculiarities of pepsin digestion may be accounted for in this way. It seems possible to explain the kinetics of an enzyme hydrolysis fairly well from either point of view. It is possible to find very close analogies on the one hand with ordinary homogeneous catalysis, and on the other hand with heterogeneous catalysis. From the theoretical side it has been stated that since most enzymes are admittedly colloidal it is not justifiable to apply the law of mass action to them. It may be pointed out, however, that the work of Perrin and of Svedberg[4] has shown that colloidal solutions obey the gas laws very well, in fact, much better than do ionized substances. Since the law of mass

[1] Arrhenius, S., Quantitative laws in biological chemistry, London, 1915.

[2] For a discussion of this question see von Euler, H., Allgemeine Chemie der Enzyme, 2nd edition, Munich, 1900.

[3] Taylor, A. E., On fermentation, *Univ. Calif. Pub.*, Pathology, 1903–07, i, 244.

[4] Svedberg, T., *Z. physik. Chem.*, 1910, lxxiii, 547; Svedberg, T. and Inouye, K., 1911, lxxvii, 145. Lewis, W. C. McC., A system of physical chemistry, i, 1918.

action can be derived from the gas laws and the two laws of thermodynamics, there seems to be every reason to suppose that colloidal solutions should obey the law of mass action. The question appears to be one of experimental fact. If it is found that enzyme reactions may be accounted for on the basis of the law of mass action there seems to be no theoretical reason to disregard this fact and attempt an explanation from the point of view of adsorption. Most of the experimental evidence at hand, however, consists of data on the kinetics of the reactions. It is a matter of experience that conclusions based on kinetics alone are exceedingly uncertain especially when, as is the case with enzymes, the equations used contain at least two constants. It seems better therefore to attack the question from another angle. It is known that various substances retard the action of enzymes and that there must therefore be some kind of a reaction between these substances and the enzyme (or the substrate). If it could be shown that this reaction conformed accurately to the law of mass action it would furnish experimental justification for the application of this law to the enzyme reaction in general, at least as far as the particular enzyme is concerned. It has been shown by the author that the equilibrium between pepsin and the products formed by its action on proteins does conform quite accurately to the law of mass action.[5] Euler and Svanberg[6] have shown that the inactivation of invertase by various crystalloid substances is due to an equilibrium which is accurately expressed by the same law. The experiments described in this paper were undertaken with the view of determining whether or not the same condition is found in the case of trypsin. As will be seen from these experiments, the reaction between trypsin and the substances which inhibit its action may be accurately accounted for by the law of mass action.

It is known that various substances inhibit the action of trypsin. Bayliss[7] found that some, at least, of the products formed by the action of the enzyme on proteins inhibited its action and also rendered it more stable. He concluded, therefore, that they combined in some

[5] Northrop, J. H., *J. Gen. Physiol*, 1919–20, ii, 471.

[6] von Euler, H., and Svanberg, O., *Fermentforschung*, 1919–20, iii, 330; 1921, iv, 142.

[7] Bayliss, W. M., *Arch. Biol.*, 1904, xi, Suppl. 261.

way with the enzyme. Bayliss considered that the amino-acids were probably responsible for this inhibiting effect. It would seem, therefore, that these substances are favorable for the purpose of the present experiments since they are also involved in the kinetics of the reaction, and are chemically well defined substances. It was found, however, that the amino-acids themselves have very little if any effect, if the effect on the pH was controlled, whereas the solution resulting from the hydrolysis of the protein by trypsin was strongly inhibitory. Such solutions were, therefore, used although it was not found possible to determine exactly what chemical compound was responsible for their reaction.

Methods Used in the Present Investigation.

Inasmuch as the quantity of an enzyme can only be determined by measuring the rate at which it reacts it is necessary to have some convenient and accurate method for following the course of the hydrolysis. In the present experiments it is also necessary to arrange conditions in such a way as to prevent any change in pH during the course of the hydrolysis. Henri and des Bancels[8] found that the hydrolysis of proteins by trypsin could be accurately followed by noting the change in the conductivity of the solution. The same method was used by Bayliss who also showed that the change in conductivity was not caused by changes in the viscosity of the solution and was parallel to the amount of non-precipitable nitrogen formed. A number of experiments were made to determine the best conditions to use for the purpose of the present experiments. It was found that at a pH of 6.2 to 6.4 no change occurred in the pH during the hydrolysis of gelatin (due probably to the fact that this is near the isoelectric point of the products formed), so that it was not necessary to use buffer solutions if the determinations were made in this range. This is a great advantage since buffers interfere with the determination of the conductivity. Gelatin was found to be the best protein since it gives clear solutions and is easily prepared in the necessary salt-free condition. Since trypsin is very unstable in solution the experiments were made at 33°C. The change in the conductivity of the solutions was followed by the change in the readings of the Kohlrausch bridge. These readings are proportional to the percentage change in the conductivity, so that the lower the original conductivity of the solution the greater will be the change in the bridge readings for a given absolute increase in the conductivity of the solution. On the other hand, the lower the original conductivity the more sensitive the solution is to the addition of traces of salts, to temperature change, etc. It was found better, therefore, to increase the conductivity of the gelatin by the addition of KCl until the solution had a specific conductivity at 33°C. of 2×10^{-3} reciprocal ohms.

[8] Henri, V., and des Bancels, L., *Compt. rend. Biol.*, 1903, lv, 563, 787, 788.

Experimental Procedure.

Preparation of the Gelatin.—Salt-free gelatin was prepared as described by Loeb,[9] *i.e.*, the gelatin was brought to the isoelectric point and then washed (except that 100 gm. were prepared at a time). The gelatin was melted, diluted to about 5 per cent and titrated to a pH of 6.3 with NaOH. It was then diluted so as to contain 2.5 gm. dry weight per 100 cc. and sufficient KCl added so that the resulting solution had a specific conductivity at 33°C. of 2×10^{-3} reciprocal ohms. A few crystals of thymol were added and the solution was kept in the ice box.

Preparation of the Trypsin.—The trypsin used in all the experiments was a sample of Fairchild's trypsin. It was prepared for use by suspending 5 gm. in 50 cc. of water and dialyzing under pressure at 6°C. for 18 hours. The solution was then filtered from the rather heavy precipitate and sufficient KCl added to bring the specific conductivity to 2×10^{-3}. This solution is very unstable and loses its activity quite rapidly even at 3°C. It was prepared fresh each day.

Determination of the Conductivity.—The apparatus used was a Leeds and Northrup Kohlrausch bridge and resistance box. The change in the bridge readings were used direct to avoid calculation. These readings are related to the actual conductivity by the formula

$$X = \frac{A}{1000 - A} R$$

in which X = resistance of solution, A = bridge reading, and R = resistance of standard resistance box. It will be seen that if the readings are always begun at the middle of the bridge (500), the first ten or fifteen points will each represent very nearly equal changes in the conductivity and may be considered as proportional to the percentage change in the conductivity. Since the conductivity increases the bridge readings will decrease. If all the solutions have the same conductivity at the beginning of the experiment and the resistance is so chosen that the bridge reading at the beginning is 500 in each case, then a change in the bridge reading of from 500 to 490 will represent the same change in the conductivity of each of the different solutions.

Type of Conductivity Cell.—The cell used is shown in Fig. 1. Fifteen such cells were made and adjusted (by warming the cement and moving the electrode) so that they all had the same constant, 3.5. The electrodes were plated with platinum black. Readings could be made with ease to half a scale division on the bridge. As little current as possible was run through the cell, although no effect due to the passage of current could be noted.

pH Determinations.—The determinations were made by the E. M. F. method.

Formol Titration.—The titration was carried out as described in a previous paper[10] by adjusting the solution to pH 7.0 with neutral red as an indicator

[9] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

[10] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 595.

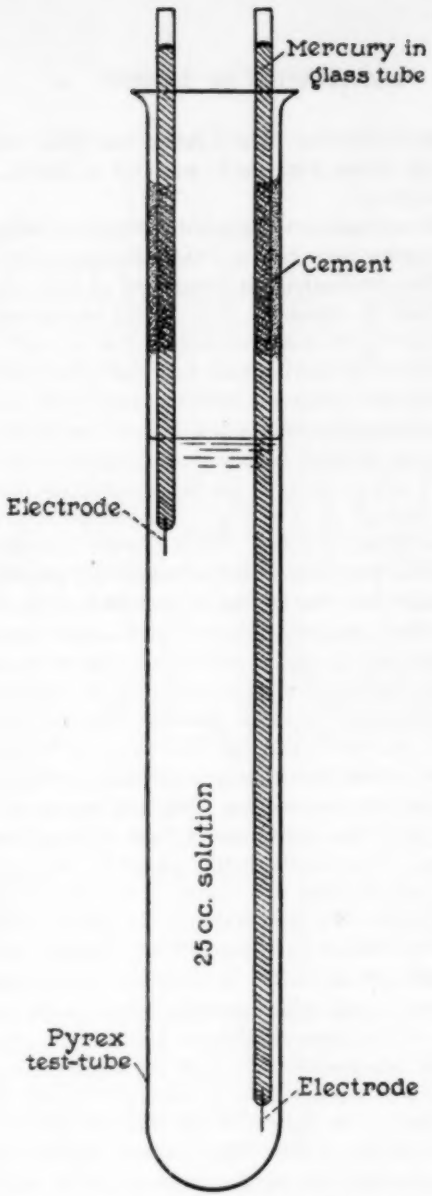

FIG. 1. Type of conductivity cell used. The cell consists of a Pyrex test-tube of about 40 cc. capacity and 20 mm. in diameter. The electrodes are made of No. 20 platinum wire sealed into fine Pyrex glass tubing. The electrodes are plated with platinum black and are 5 mm. long. The tubes holding the electrodes are fastened to the sides of the test-tube with de Kotynsky cement and are then filled with mercury. The distance between the electrodes is so adjusted that all the cells have the same constant; *i.e.*, when filled with the same solution they all read the same.

before adding the formaldehyde. The solution was then titrated to 8.2 with thymol blue. The titrations were made with 0.1 N NaOH. The end-points are both accurate to 0.05 cc.

Technique of the Determination.—The gelatin solution is melted, 25 cc. pipetted into a series of the conductivity cells and the cells suspended in the water bath at 33° ± 0.01°C. The conductivity is determined at intervals until it becomes constant (usually about 20 minutes). 1 cc. of the trypsin solution (previously warmed to 33° and having the same conductivity) is then added. The solution is then thoroughly mixed by sucking back and forth three times in a warm dry 15 cc. pipette. It is necessary to avoid air bubbles and to be sure that the solution is well mixed. Irregular results can nearly always be traced to incomplete mixing. The conductivity of the solution is then read at intervals so that readings are obtained at every 1.5 or 2 divisions on the bridge, until the reading is 485 or less (corresponding to a decrease of 15 points). The elapsed time is calculated from the time at which the trypsin is added. Since in order to obtain the elapsed time it is necessary to make a great many subtractions it is a great convenience to use a clock which is divided into hundredths of an hour instead of minutes. If the trypsin solution has been carefully adjusted to the same temperature and conductivity as the gelatin and the mixing carried out without change of temperature or the formation of air bubbles, it will be found that the readings form a perfectly smooth curve. It frequently happens, however, that the first reading (before the trypsin is added) does not fall on the same curve as the subsequent readings. In this case the curve is extrapolated back from the first reading after the trypsin is added in order to find the zero reading. Since, as was stated above, the curve is perfectly smooth when the experiment is done with sufficient care this procedure seems justified. The results should always fall on a smooth curve after the first 0.05 hour (corresponding to a change of from 0.1 to 1.0 on the bridge). The results are then plotted on a large scale and the time necessary for the reading to change 10 points determined by interpolation. This corresponds to an actual change in the conductivity of 0.0782×10^{-3} reciprocal ohms and is less than 10 per cent of the total change which can be effected by the trypsin under these conditions. Owing to the large number of experiments, the individual time curves from which the time necessary to cause the 10 points change is determined will not be given, but only the time interval interpolated from these curves. The points lie so close together on the curve that there is little or no possibility of arbitrary adjustment of the curve. The elapsed time is, therefore, a direct experimental determination. In the few cases where it was possible to draw more than one curve through the experimental points, the extreme values for the interpolated time have been given.

Comparison of the Course of the Reaction as Followed by the Formol Titration of the Solution and by the Change in Conductivity.

The most significant determination as regards the hydrolysis of proteins is the increase in amino or carboxyl groups. It was found that under the conditions adhered to in these experiments the increase in carboxyl groups is directly proportional to the increase in the conductivity. This is shown in Fig. 2. The values obtained by the conductivity method therefore represent the actual course of the hydrolysis. This is not true under all conditions. It was found that at other ranges of acidity the two determinations are not parallel. The change in conductivity is also dependent on the alkali used to bring the gelatin to the required pH. It is greatest in ammoniacal solution and may even decrease instead of increase in concentrated phosphate solutions.

Since the rate of hydrolysis is to be used to determine the amount of trypsin present it is necessary to have some method of expressing the velocity of the hydrolysis. This value should be independent of the stage of the hydrolysis at which the determination is made since otherwise it would evidently be possible to obtain a series of values depending on what stage of the hydrolysis was chosen. The most satisfactory figure for such purposes is of course the constant obtained by substituting the observed values in some equation such as the monomolecular reaction equation. The hydrolysis as carried out in these experiments, however, does not follow accurately any of the simple reaction formulæ so that this method cannot be used. It was found that the reciprocal of the time required to cause a definite change was very nearly directly proportional to the concentration of trypsin; *i.e.*, $QT = K$. Where Q is the concentration of trypsin, T the time required to cause a given small change, and K is a constant. As Arrhenius[1] has pointed out, this is a general property of enzymes even though they do not follow the formula for a monomolecular reaction. This rule has been found to hold for trypsin by Taylor,[3] Henri,[8] Vernon,[11] Hedin,[12] and Bayliss,[7] whereas Grützner[13] states that the rule does not hold, but that the reciprocal of the time

[11] Vernon, H. M., *J. Physiol.*, 1904, xxx, 330.
[12] Hedin, S. G., *J. Physiol.*, 1905, xxxii, 468.
[13] von Grützner, P., *Arch. ges. Physiol.*, 1911, cxli, 63.

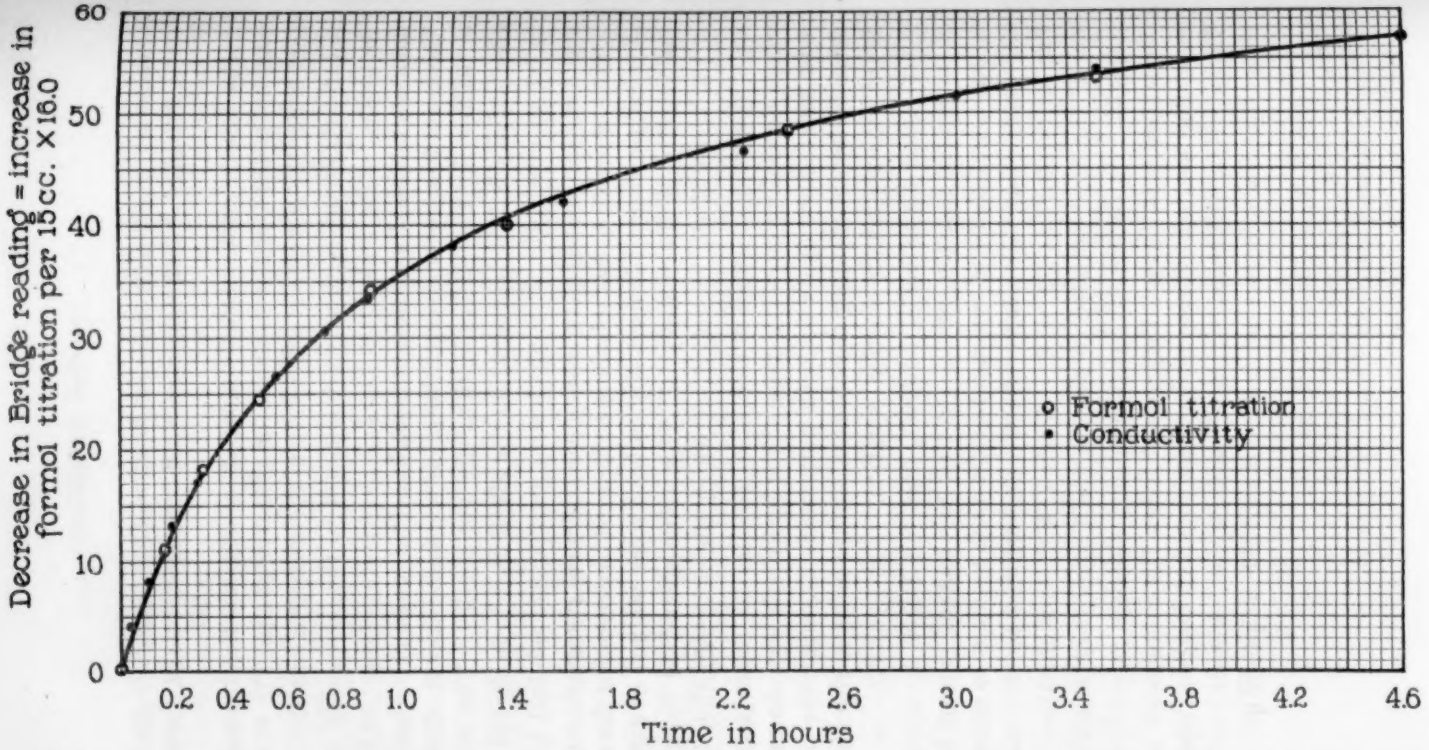

FIG. 2. Comparison of the increase in conductivity (as measured by the decrease in the bridge readings), with increase in the formol titration of the solution. The formol titration was made on 15 cc. of solution. The results have been plotted to the same scale by multiplying the formol titration by 16. The ordinates therefore represent the decrease in bridge reading or the formol titration (expressed as cc. 0.10 N NaOH) per 240 cc. of solution.

increases more rapidly than the amount of enzyme taken. It will be shown below that the results depend on the purity of the enzyme and protein solutions used. In the case of purified trypsin and

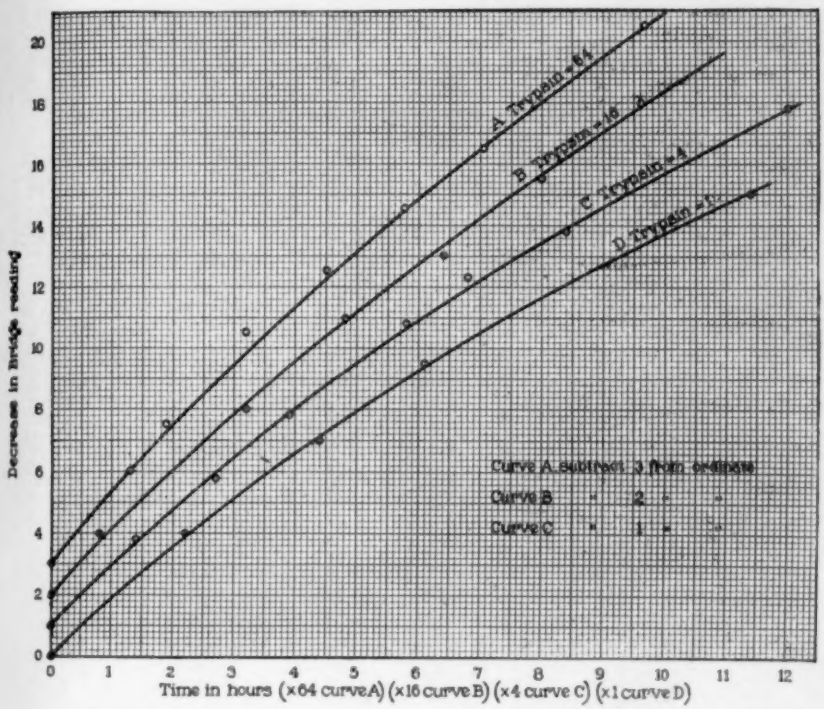
Decrease in bridge reading
Time in hours (×64 curve A) (×16 curve B) (×4 curve C) (×1 curve D)
A Trypsin = 64
B Trypsin = 16
C Trypsin = 4
D Trypsin = 1
Curve A subtract 3 from ordinate
Curve B " 2 " "
Curve C " 1 " "

FIG. 3. Influence of the concentration of trypsin on the rate of digestion. 25 cc. gelatin plus 1, $\frac{1}{4}$, $\frac{1}{16}$, $\frac{1}{64}$ cc. of trypsin solution. The change in the conductivity determined at intervals as shown in the figure. pH 6.4, temperature 33°. In order to give all the results in one figure the time units for each curve have been made proportional to the amount of trypsin added; *i.e.*, in the solution containing a relative trypsin concentration of 64 (1 cc.) the observed time has been multiplied by 64. To avoid confusion the ordinates (bridge readings) have been increased by 1 (Curve *C*), 2 (Curve *B*), and 3 (Curve *A*).

gelatin solutions the velocity (reciprocal of the time) is nearly directly proportional to the amount of enzyme taken within the limits that can be experimentally worked with. This fact is shown in Fig. 3. In plotting this figure the time units for each concentration of trypsin

have been multiplied by the relative concentration of trypsin used in obtaining the curve corresponding to that concentration. It will be seen that if the formula $QT = K$ is correct, the resulting curves should be identical (or parallel if plotted as in the figure). As the figure shows this is not quite true. The curves for the concentrated solutions do not drop off as rapidly as those for the dilute. That is the

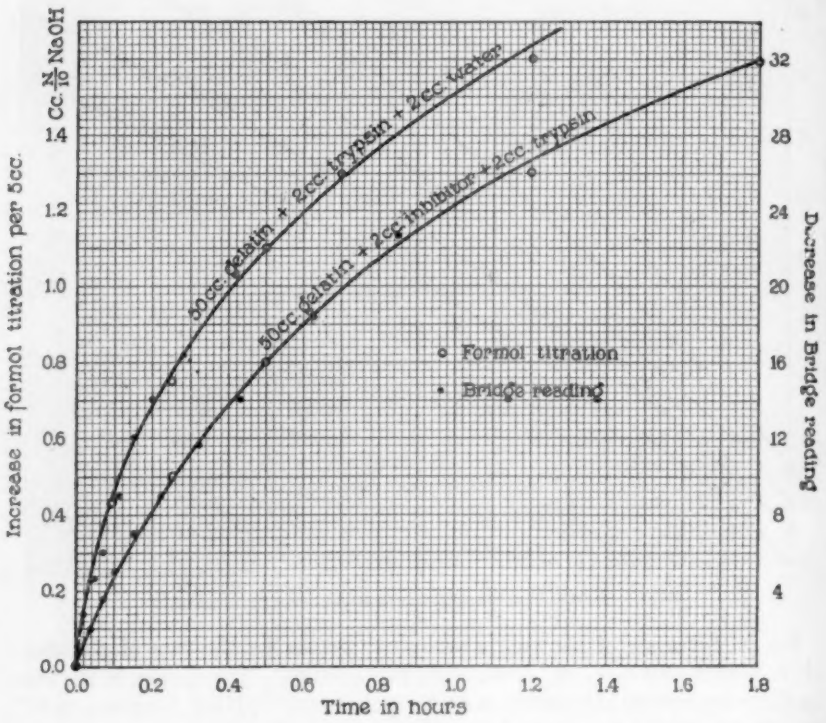

FIG. 4. Comparison of the retardation due to the presence of the "inhibitor" as determined by the formol titration or by the conductivity method.

velocity as measured by the reciprocal of the time increases more rapidly than the trypsin concentration. This is due, as will be shown later, to two causes. First, the products formed by the action of the trypsin inhibit its action, and since at any given stage of the hydrolysis the concentration of the products in the different solutions will be the same but the concentration of trypsin different, the percentage

inhibition of the solution containing a small amount of trypsin will be greater than in that containing a larger amount of trypsin. Second the trypsin is constantly becoming irreversibly inactivated. The amount of this inactivation is proportional to the time and is therefore proportionately greater in the dilute solutions since these require a longer time to cause the change used as the end-point. This effect is much more noticeable at a higher temperature, as would be expected.

TABLE I.

Relative concentration of trypsin.	Time required to change 10 points observed.	(QT = 550) calculated.	QT
	hrs. × 10^2		
64	7.5	8.5	480
	7.3		467
32	17.5	17.0	560
	16.0		510
16	34	34.0	540
	35		560
8	80	68	640
	82		656
4	155	136	620
	160		640
2	300	272	600
	250		500
1	660	542	660
	680		680

Both these effects are in the same direction and both become more noticeable the longer the hydrolysis proceeds, as Bayliss[7] found. The experiment was made in duplicate and was run with intermediate trypsin concentrations which are not plotted. Table I gives a summary of the whole experiment. The table shows that the values found for the amount of trypsin are within about 10 per cent of the expected values over the entire range of the experiment and much closer for smaller variations of the trypsin concentration. This dif-

ference is not much greater than the experimental error. The experiment was repeated several times and it was found that in general the reciprocal of the time required to cause a change of 10 points in the bridge reading was directly proportional, within the experimental error, to the amount of trypsin taken. This value has therefore been used to express the amount of active trypsin present in the solution. The unit of trypsin used in these experiments may be defined as that quantity which when added to 25 cc. of gelatin solution having a pH of 6.3 (adjusted + NaOH) and a specific conductivity of 2×10^{-3} (adjusted + KCl) will cause a change in the bridge reading of 10 points (500 − 490) in 1 hour at 33°C. (equivalent to an increase in the conductivity of 0.0782×10^{-3} reciprocal ohms).

Properties of the Trypsin Used.

It has been stated by Vernon[11] and others that "trypsin" may be separated more or less into a number of enzymes some of which attack gelatin more rapidly than other proteins and some of which act best on peptones. If this is the case the experiments are evidently complicated by another factor in addition to the many already present. Many experiments were made but no evidence could be found to show the presence of such enzymes in the sample of trypsin used in these experiments. The trypsin was treated in a number of different ways and the relative velocity with which it hydrolyzed gelatin or peptone compared before and after the treatment. (The peptone was prepared by the action of pepsin on gelatin sulfate solution. The acid was then removed with barium and the solution made alkaline with NaOH.) A summary of these experiments is given in Table II. It will be seen that there is no evidence for the existence of any special "peptonase" or "gelatinase" in the sample of trypsin used in these experiments.

Effect of Dialysis on the Trypsin.

It has already been stated that the trypsin was purified by dialysis. The effect of this is shown in Table III. It will be seen that the activity of the trypsin is more than doubled by the process and that the solution obtained in this way contained only about 0.02 gm. per cc. of total solids.

TABLE II.

Influence of Treatment of Trypsin on Ratio of Peptonase and Protease.

Gelatin, 2 per cent, pH 6.2, specific conductivity 0.0022. Gelatin peptone, gelatin hydrolyzed by trypsin, 2 per cent, pH 6.2, specific conductivity 0.0030.

Treatment of trypsin.	$T_0 - 10$ gelatin.	$T_0 - 10$ gelatin peptone.	$\frac{T_0 - 10 \text{ gelatin peptone.}}{T_0 - 10 \text{ gelatin}}$
	hrs. × 10^2	hrs. × 10^2	
1 per cent Fairchild's trypsin solution.	25	22	1.14
	29	24	1.21
	26	24	1.09
	28	28	1.00
	34	32	1.07
	35	30	1.17
Average			1.11
10 gm. trypsin dissolved in 100 cc. water, precipitated with 50 cc. alcohol. Precipitate = 0.1 gm. dissolved in 20 cc. H_2O.	30	24	1.24
	27	22	1.22
	29	28	1.04
	32	31	1.04
	37	31	1.19
Average			1.14
50 cc. 10 per cent trypsin dialyzed 18 hours at 6°C., brought to pH 6.8 + NaOH, then left 3 hours at 38°C. (about 98 per cent inactivated).	28	27	1.0
	25	33	0.7
	23	32	0.7
Average			0.8
Control.	40	46	0.86
	35	36	0.90
	37	36	0.90
Average			0.88
50 cc. 10 per cent trypsin dialyzed 18 hours at 6°C., brought to pH 4.2 with HCl and kept at 38°C. for 1 hour, about 80 per cent inactivated.	25	22	1.13
	25	21	1.20
	26	26	1.00
	24	24	1.00
Average			1.08
Control.	39	33	1.18
	48(?)	37	1.27
	50	44	1.10
	45	46	0.98
Average			1.13

TABLE III.

Dialysis of Trypsin.

2.5 gm. of trypsin in 25 cc. water, dialyzed under pressure 18 hours, and filtered.

	Time required to change 10 points.	Trypsin per cc.	Dry weight 1 cc.	Trypsin per gram.	Formol titration.	pH
	hrs. × 10^3	*units*	*gm.*	*units*	*cc.* 0.1 N NaOH	
Before dialysis	5.0	20	0.10	200	3.3	
After dialysis	10.0	10	0.02	500	0.4	6.2 to 6.4

Preparation of the Inhibiting Solution.

Bayliss[7] found that glycine and other amino-acids as well as the digested protein solution inhibited the action of trypsin. Several amino-acids were tried but with negative results except in concentration very much higher than could possibly be present from the protein. In such high concentration the determination becomes uncertain, owing to the high conductivity of the solution. Bayliss' experiments were made at a time when the determination of the hydrogen ion concentration was a difficult matter and it seems possible that the results he obtained were due to changes in the pH caused by the addition of the amino-acids rather than to an effect on the enzyme. A solution of casein or gelatin which has been hydrolyzed by trypsin does show marked inhibitory effects, however. Owing to the manner in which these experiments were made it was necessary to have the solution nearly salt-free and in a concentrated form so that the volume change on adding it to the gelatin would be small. It is also necessary to be sure that there are no products left in solution that can be further acted on by trypsin. It was found that a solution having strong inhibitory powers could be made from either gelatin or casein by the following method.

Preparation from Gelatin.—3 liters of 1.5 per cent gelatin were titrated to a pH of 9.0 with $Ba(OH)^2$ and 10 cc. dialyzed trypsin and a few crystals of thymol added. The solution was kept at 23° until no further increase in the formol titration could be noted. The titration increases about 700 per cent. The solution was then titrated to a pH of 6.3 with sulfuric acid and the barium sulfate

filtered off. The solution was then evaporated in vacuum to 60 cc. 1 cc. of this solution contained the equivalent of 0.7 gm. of gelatin and had a formol titration of 16 cc. of 0.1 N NaOH.

Preparation from Casein.—200 gm. of commercial casein were dissolved in 3 liters of water and precipitated by the addition of sulfuric acid. The precipitate was washed in water, suspended in 1 liter of water and heated to boiling. $Ba(OH)_2$ was then added until the supernatant liquid had a pH of 9.0. The solution was cooled and 5 cc. dialyzed trypsin added and the solution kept at 23° for 10 days. It was then filtered, the filtrate titrated to pH 6.3 with sulfuric acid, evaporated in vacuum to 100 cc., and precipitated by the addition of 800 cc. of 95 per cent alcohol. A gummy precipitate forms which consists of higher products which are still acted upon by trypsin. The filtrate is evaporated *in vacuo* to a thick syrup to remove the alcohol and taken up in 50 cc. water. The solution so obtained is a clear yellowish syrup. It is not further acted upon by trypsin and contains no active trypsin. The formol titration per cubic centimeter is equivalent to 15 cc. of 0.1 N NaOH. This solution was then accurately adjusted to pH 6.3 with HCl and to a specific conductivity of 2×10^{-3} reciprocal ohms by the addition of a small amount of KCl. The experiments described in this paper were all made with the solution prepared in this way from casein. It is referred to as inhibiting solution or inhibitor.

Properties of the Inhibiting Solution.

It had already been stated that no effect could be noted if amino-acids were added to the trypsin, unless very much higher concentrations were used than could be furnished by digestion of the protein.

Glycine, alanine, tryptophane, leucine, tyrosine, arginine, proline, and histidine were tested alone and in combination. It was also found that gelatin or casein which had been completely hydrolyzed by either acid or alkali was without effect. The results of some of these experiments are shown in Table IV. The experiment shows that the inhibiting substance is not formed in the acid or alkali hydrolysis of gelatin or casein, and that it is dialyzable. The evidence is not sufficient to prove that the inhibiting substance is a specific result of trypsin hydrolysis since the acid and alkali hydrolyses were only tested when the reaction had continued far beyond the stage reached by trypsin hydrolysis. It may be mentioned, however, that it has been found by an entirely different method that the hydrolysis of gelatin by acids or alkalies or trypsin in the early stages follows a different course and must give rise to different products in each

case.[14] The action of pepsin gives rise to substances which inhibit pepsin but which are themselves attacked by trypsin.

TABLE IV.

Retarding Effect of Various Solutions of Hydrolyzed Gelatin or Casein on the Action of Trypsin.

25 cc. of gelatin pH 6.2, specific conductivity 2×10^{-3}, plus 1 cc. of solution noted. 1 cc. of trypsin added and time required to cause a change of 10 points (bridge reading) in the conductivity determined. In each case the amount of hydrolyzed protein added is equivalent to 0.2 gm. of the original protein.

Solution.	Time required to change 10 points.	
	Control.	+ 1 cc. solution.
	hrs. × 10^2	*hrs.* × 10^2
(*a*) Gelatin hydrolyzed by trypsin	22	45
(*b*) Casein hydrolyzed by trypsin	15	36
(*c*) Casein completely hydrolyzed, by acid	25	27
		22
(*c*) Casein completely hydrolyzed, by alkali	40	44
		40
(*d*) Gelatin completely hydrolyzed, by acid	17	20
	19	18
(*d*) Gelatin completely hydrolyzed, by alkali	34	36
	30	38
(*e*) Solution (*a*) after 24 hrs. dialysis	16	15
	14	15
Solution (*b*) after 24 hrs. dialysis	28	29
	24	26

Method of Determining the Amount of Retardation.

It has already been shown that the rate of hydrolysis of gelatin alone as determined by the change in conductivity is identical with that found by the formol titration. Fig. 4 shows that this is also the case when the hydrolysis has been retarded by the addition of inhibitor

[14] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 57.

solution. The figure and Table V show that the retardation is nearly independent of the stage of hydrolysis compared for the first part of the reaction but then becomes relatively less. The retardation may therefore be calculated from the velocity of the reaction provided the early part of the reaction curves are compared. (It has been found by Simons[15] in Nelson's laboratory that this is not the case with invertase.) The result found with trypsin, namely, that the retarding effect of the inhibiting solution becomes less as the reaction proceeds, is exactly what would be expected if it is supposed that the inhibitor combines with the enzyme to form an inactive com-

TABLE V.

Influence of Inhibitor on Rate of Hydrolysis Followed by Formol Titration and by Conductivity.

Temperature, 33°C. 2.0 per cent gelatin, pH 6.2 + NaOH. Specific conductivity, 2.2×10^{-3}.

Increase in formol titration per 5 cc. solution. (*a*)	Decrease in bridge reading.	Time required to cause increase in formol titration given under (*a*).		
		Control.	Inhibitor.	$\frac{T \text{ inhibitor}}{T \text{ control}}$.
cc. 0.1 N NaOH	*units*	*hrs.*	*hrs.*	
0.3	6	0.06	0.13	2.17
0.5	10	0.12	0.26	2.16
1.0	20	0.41	0.72	1.70
1.5	30	1.00	1.54	1.54

pound and that more of the inhibiting substance is formed during the hydrolysis (or some of the trypsin destroyed). The retarding effect of the inhibiting substance formed during the reaction will evidently be much less in the solution that already contained the inhibitor than in the solution which contained only free trypsin. The inhibitor acts just as a "buffer" solution for regulating the hydrogen ion concentration, except that in this case it is the enzyme that is "buffered." In fact the same experiment may be performed by following the hydrolysis of gelatin with a weak as compared with a

[15] Simons, L. S., Dissertation, Columbia University, 1921.

strong acid at about the same pH.[16] This mechanism will evidently lead to the result that the solution containing inhibitor will be relatively more active compared to the control solution as the hydrolysis proceeds, which is the experimental fact. The same fact has been noted in immunology in comparing the action of free toxin and of a mixture of partially neutralized toxin. Bordet[17] considers that it is the degree of activity of the total amount of toxin that is affected by the antitoxin and not the concentration of free toxin. His experiment is similar to the trypsin experiment just discussed, inasmuch as he found that a small amount of free toxin reacts at first more rapidly than a mixture of toxin-antitoxin, but that as the reaction proceeds, the mixture becomes relatively much more efficient. Bordet's explanation will not explain the results with trypsin quantitatively while the assumption of the formation of an inactive compound between the trypsin and inhibitor allows all the peculiarities of the reaction to be calculated. It does not follow, of course, that the toxin-antitoxin reaction is the same as the trypsin-inhibitor reaction, but it seems that the same explanation will apply qualitatively to both.

[16] If the pH of the solution containing the strong acid is slightly lower than that of the weak acid, the rate of hydrolysis will at first be greater in the solution containing the strong acid. The rate of hydrolysis in this solution will decrease rapidly, however, since the concentration of hydrogen ions will be diminished by the products of hydrolysis—just as is the concentration of free trypsin in the present experiment. The rate of hydrolysis in the weak acid solution, however, will remain nearly constant since the hydrogen ions which combine with the products formed will be replaced by the dissociation of more of the weak acid. The rate of hydrolysis in the weak acid solution will therefore constantly increase as compared to the rate of hydrolysis in the strong acid solution. An example of such an experiment is given in a preceding paper (Northrop, J. H., *J. Gen. Physiol.*, 1920–21, iii, 725, Fig. 3, Curves III and IV).

[17] Bordet, J., Immunité, Paris, 1920, p. 530.

THE INACTIVATION OF TRYPSIN.

II. The Equilibrium between Trypsin and the Inhibiting Substance Formed by Its Action on Proteins.

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The Equilibrium between Trypsin and the Inhibiting Substance.

The experiments already described[1] show that it is possible to prepare a solution by the action of trypsin on a protein which inhibits the action of trypsin. It has also been shown that the amount of this retardation can be quantitatively measured by comparing the times necessary to cause a given small change in the conductivity of the gelatin solution under the conditions adhered to.

A number of hypotheses may be proposed that will account qualitatively for this retardation. The simplest would be to assume that the inhibiting substance combined with trypsin to form a compound that is inactive and that the activity of the solution is proportional to the concentration of free trypsin remaining in the solution. It has already been shown that if pure trypsin and protein is used the velocity of hydrolysis is proportional to the amount of trypsin taken. This is the experimental fact and is independent of any hypothesis as to the kinetics of the reaction. If it is further assumed that the equilibrium is governed by the law of mass action it is possible to test this hypothesis quantitatively. This has been done in the following experiments.

Influence of the Order of Mixing and of the Time of Standing on the Equilibrium.

Since in most of the experiments the retarding effect of the inhibiting solution has been determined by adding the solution to the gelatin

[1] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 227.

and then adding the trypsin and determining the rate of hydrolysis at once, it is necessary to know whether or not the order of mixing the solutions or the time during which the trypsin has been allowed to react with the inhibiting substance has any influence on the result. Table I is a summary of experiments planned to answer this question. It shows that the order of mixing and the time during which the trypsin and inhibiting solution are left together has no effect on the final result. That is, the equilibrium between the trypsin and the inhibitor must be reached practically instantaneously and be quantitatively and instantly reversible. (This is only true if the experiment

TABLE I.

Effect of Order of Mixing and Time of Standing.

SOLUTION.	Time to change 10 points after hours at 6°C.	
	0 hours.	1.0 hours.
	hrs. × 10^3	*hrs.* × 10^3
(1) 25 cc. gelatin + 1 cc. trypsin	15 16	16
(2) 25 cc. gelatin + 1 cc. inhibitor + 1 cc. trypsin added after interval shown	21 25	21 20
(3) 25 cc. gelatin + 2 cc. mixture of 5 cc. inhibitor + 5 cc. trypsin. (Mixture allowed to stand as noted and 2 cc. then added to gelatin)	22	20

is made under such conditions that the control trypsin solution—without inhibitor—remains unchanged during the course of the experiment. If this condition is not fulfilled, the results depend entirely on the length of time and on the temperature at which the trypsin-inhibitor solution has been allowed to stand.)[2]

Influence of the Gelatin Concentration.

The retardation could be qualitatively accounted for by assuming that the inhibiting substance combines with the gelatin instead of

[2] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 261.

with the enzyme. If this were true the retardation should be less the greater the concentration of gelatin, provided the relative amount of inhibitor to trypsin were kept the same. Table II shows that this is not the case. The amount of retardation is independent of the concentration of gelatin used. The result is confirmed by the experiment discussed below in which the concentration of inhibitor is kept the same and the concentration of trypsin varied. If the inhibitor combined with the gelatin, the resulting solution would act as though

TABLE II.

Influence of Gelatin Concentration on Retardation of Hydrolysis by Inhibiting Solutions.

Control, 25 cc. gelatin of concentration noted + 1 cc. trypsin + 1 cc. 0.01 N NaCl. *Solution*, 25 cc. gelatin of concentration noted + 1 cc. trypsin + 1 cc. inhibiting solution. Temperature 33°C. Specific conductivity of all solutions 1.2×10^{-3} (adjusted with NaCl, and pH of 6.0 adjusted with NaOH).

$\frac{\text{Time required for 10 points change with 1 cc. trypsin}}{\text{Time required for 10 points change with 1 cc. trypsin + 1 cc. inhibitor}}$ in gelatin concentrations of

1 per cent.	2 per cent.	4 per cent.	8 per cent.
66	65	67	
67	65		
70	70		73
75	75	75	76
76	70	73	71
	75	73	72
Average 70.8 ±1.8*	70.0 ±1.4*	72.0 ±1.2*	73.0 ±0.7*

* Average deviation of the mean.

a lower concentration of gelatin had been used and the velocity would still be directly proportional to the amount of trypsin added to the solution. This is not the case. The fact that the inhibiting solution renders trypsin more stable (when no protein is present) also shows that it combines with the trypsin.

Influence of Inactivated Trypsin.

It was found in the case of pepsin[3] that pepsin inactivated by alkali took part in the equilibrium just as does the active pepsin

[3] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 471.

(pepsin inactivated by heat does not act in this way). If it should be found that inactive trypsin also took part in the equilibrium it is evident that the experiments are complicated by an additional factor that is very hard to control since there is no independent method for determining the amount of inactive trypsin. In order to determine this point the experiments described in Table III were performed. They show that the inactive trypsin does not take part in the equilibrium. The calculated results are obtained from the law of mass action as described below.

TABLE III.

Addition of Inactive Trypsin.

2 per cent gelatin pH 6.2, specific conductivity 2×10^{-3}. Trypsin, 10 per cent, dialyzed, time to change 10 points = 0.10 hours = 10 units per cc. Inactivated at 65°C. for 2 hours. Active trypsin, 10 per cent, dialyzed, diluted one-third. $P = 10$. $K' = 2.8$.

Active trypsin.	Inhibitor.	Inactive trypsin.	Time to change 10 points.	Q		
				Observed.	Calculated.	
					Inactive no effect	Inactive enters equilibrium.
cc.	*cc.*	*cc.*	*hrs.* $\times 10^2$			
1	0	0	36	2.7	2.7	
			39			
1	0.5	0	90	1.1	1.2	1.2
			87			
			90	1.1	1.2	1.9
1	0.5	1	80	1.2		
			95	1.05		

Effect of Adding Increasing Amounts of Inhibitor to a Constant Quantity of Trypsin.

The results of a series of experiments to determine the effect of adding increasing concentrations of inhibitor to the same amount of trypsin are given in Table IV and Fig. I. The experiments were carried out by adding the noted amount of inhibiting solution to 25 cc. of gelatin at 33°C. All the solutions had the same pH, which re-

TABLE IV.

Effect of Increasing Amounts of Inhibitor on the Rate of Hydrolysis.

25 cc. gelatin pH 6.2; specific conductivity 2×10^{-3}. 1 cc. trypsin (10 per cent dialyzed). $V = 28$ cc. $P = 10$. $K' = 2.8$.

Cc. inhibitor $= \frac{d}{10}$	Time to change 10 points.	Trypsin. Free $\frac{1}{T} = Q$ observed.	Trypsin. Combined. $E - Q$ observed.	Trypsin. Calculated. Q	$\frac{Qd}{10}$	Trypsin combined per 0.125 cc. of inhibitor added.
	hrs. $\times 10^2$					
0	44	2.3	0	2.3		
	48					
0.125	52	1.9	0.4	1.81	0.24	1st 0.125 cc. 0.40
0.25	64	1.56	0.74	1.45	0.39	2nd " 0.34
0.50	90	1.10	1.20	1.00	0.55	3+4th " 0.18
1.00	155	0.65	1.65	0.58	0.65	5 to 8th " 0.11
2.00	300	0.33	1.97	0.32	0.66	9 to 16th " 0.03

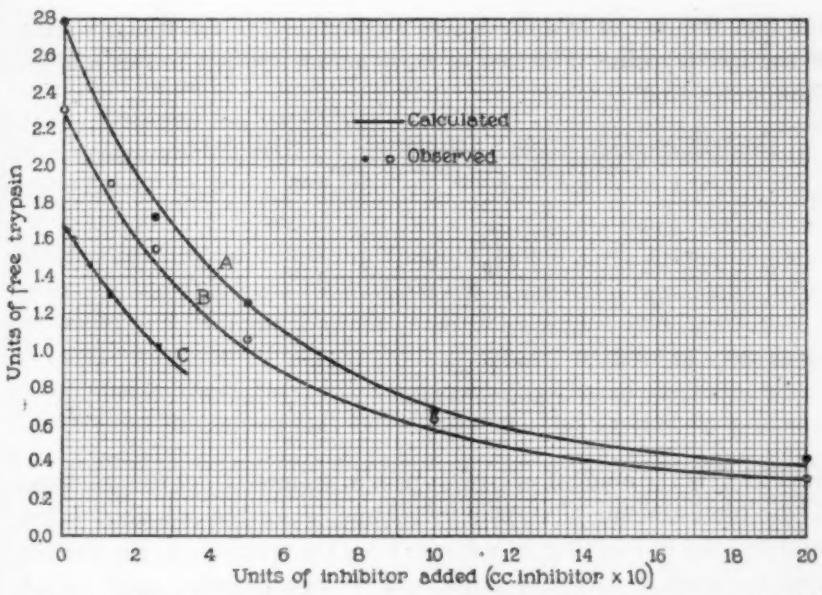

FIG. 1. Effect of adding increasing amounts of inhibitor to trypsin solutions of different strengths. The solid curves are the calculated values, and the points, the observed units of active trypsin present. (This is taken as the reciprocal of the time in hours necessary to cause a change in the bridge reading of 10 points.)

mained constant at 6.3, and the same initial conductivity. 1 cc. of trypsin solution, also of the same pH and conductivity, was then added in each tube and the time required to cause a change of 10 points in the bridge reading determined as already described. The experiments show that the addition of the first cubic centimeter of inhibiting solution has a much greater effect than the subsequent ones and that the effect constantly diminishes and becomes apparently asymptotic. This is evidently the cause of the phenomenon noted earlier that the rate of hydrolysis of a solution already containing some inhibiting substance is less rapidly retarded during the progress of the reaction than one which contains only "pure" trypsin. This is in qualitative agreement with the result predicted by the law of mass action. In order to apply this law quantitatively we may proceed as follows. It has been assumed that the equilibrium was expressed by the equation

$$\text{trypsin} + \text{inhibitor} \rightleftarrows \text{trypsin-inhibitor}$$

and that the rate of hydrolysis was proportional to the concentration of the free trypsin. The law of mass action applied to this equilibrium states that

$$\frac{\text{Concentration of free trypsin} \times \text{Concentration of free inhibitor}}{\text{Concentration of trypsin-inhibitor}} = \text{a constant}$$

or

$$\frac{\frac{Q}{V} \cdot \left[\frac{d-(E-Q)}{V}\right]}{\frac{E-Q}{V}} = K$$

which may be written

$$\frac{Q\,[d-(E-Q)]}{(E-Q)} = KV = K' \qquad (1)$$

in which Q is the amount of free trypsin in volume, V, of the solution; E, the total amount of trypsin in volume, V, of the solution; d, the total amount of inhibitor in volume, V, of the solution; K, the equilibrium constant in arbitrary units; and K', new constant equal to the product of the equilibrium into the volume. d will evidently be proportional to the number of cc. of inhibiting solution added and if there are P units of inhibitor per cc. of inhibiting solution, $d = P$ cc.

Solving this equation for Q we find that

$$Q = \pm \sqrt{\left(\frac{d-E+K}{2}\right)^2 + KE} - \frac{d-E+K}{2}$$

and since it is assumed that the velocity of the reaction is proportional to Q

$$\frac{dx}{dt} \propto \frac{\Delta X}{\Delta T} \propto \frac{I}{T} \propto Q \quad \text{and} \quad \frac{T_1}{T_2} \propto \frac{Q_2}{Q_1}$$

All the values in the above equations are known (in arbitrary units) except d and K. If more than one experiment is made it is therefore possible to solve for these two values, and then compare the calculated and observed values for Q. When this is done it is found that below a certain value for d, K is negative while above a certain limit the value of $\frac{K}{d}$ becomes constant owing to the fact that one of the terms of the equation becomes negligible when d is too large. It is also found that the constancy of K is very sensitive to experimental error (as was to be expected since it depends on the difference between two experimental values), so that a comparison of the observed and calculated values of Q is a better test of the formula than the constancy of K. Between the two limits for the values of d there are several values all of which give values of K which permit the calculation of the experimental results. The smallest of these has been taken. This gives a value for K of 0.1 and for P (in the particular inhibiting solution studied) of 10, both expressed in the same arbitrary units (*cf.* Euler and Svanberg[4] for a discussion of the same equilibrium with invertase, and Northrop[3] in the case of pepsin). It will be noted that the expression for the equilibrium as used in this form contains two arbitrary constants; *i.e.*, it is necessary to make two determinations before the others can be calculated. The agreement between the calculated and observed values is close enough to leave little doubt that the formula correctly expresses the facts, but the presence of two constants renders it possible that the agreement is accidental. If this were the case we should expect to find that it was necessary to use different values for K and d in each set of experiments. This is, however, not the case. All the experiments were found to agree

[4] von Euler, H., and Svanberg, O., *Fermentforschung*, 1920, iii, 330; 1921, iv, 142.

with a value for K of 0.1 as found above, and all done with the same inhibiting solution to agree with the same value of P (or d) as well. Several experiments were made which gave apparently regular results for which, however, it was impossible to find any satisfactory value for K and d. In every case of this kind it was found that the inhibiting solution used contained either active trypsin or some substance which could still be acted on by trypsin. In applying the formula, it is assumed, of course, that the only trypsin present is that in the trypsin solution added and that the substrate concentration is the same in all tubes. These experiments seem to show that the formula is not of such a general character as to fit any regular curve. As would be expected, different inhibiting solutions required different values of P. The results shown in the figure were all calculated from the same values of K' which were obtained from the first part of the experiment in Curve A. All the other results were calculated before the experiment was done, as were those described later in which the conditions were varied in other respects. The figure shows that the calculated and experimental results are identical.

Column 6 in Table IV contains the values for Qd; *i.e.*, the product of the free trypsin into the total amount of inhibitor. It will be seen that this value increases at first but becomes constant as d becomes large with respect to Q. Q therefore becomes inversely proportional to d. This may also be predicted from the mass action expression, and, as Arrhenius[5] pointed out, is the condition that leads to Schütz's rule. The steps in the derivation are as follows:

Equation (1) may be written

$$Q = \frac{K(E - Q)}{d - (E - Q)}$$

as d increases the term $(E - Q)$ increases and approaches the constant value E so that $K(E - Q)$ approaches a constant value. If d is large compared to E the term $(E - Q)$ may be neglected in the denominator and the equation written

$$Q = \frac{K''}{d}$$

or Q, the amount of active trypsin is inversely proportional to the amount of inhibitor, which is the experimental result referred to

[5] Arrhenius, S., *Medd. Kong. Vetsakad. Nobelinst.*, 1908, i.

above (therefore Qd becomes constant). Since the velocity of hydrolysis is proportional to Q and to the concentration of substrate the differential expression for the course of hydrolysis would be $\frac{dx}{dt} = QA$, where A is the amount of substrate. Substituting $\frac{K''}{d}$ for Q we have

$$\frac{dx}{dt} = \frac{K''A}{d} \qquad (2)$$

Since d, the inhibiting substance, is the same as x, the products of hydrolysis, we may substitute x for d. A may be considered a constant for the first few per cents of the hydrolysis. In any case, although A is decreasing, the term $(E - Q)$ (which has also been considered a constant in the numerator) is increasing so that the product of the two will be more nearly constant than either of the two quantities themselves. It is this product that really enters into the equation. Substituting x for d in equation (2) and integrating we obtain

$$\int x\,dx = \int K''\,A\,dt \text{ or } \tfrac{1}{2}x^2 = K_3\,T \text{ or } x = K_4\sqrt{T}$$

That is, x, the products formed, is proportional to the square root of the elapsed time. This derivation makes it clear that Schütz's rule will only hold when the concentration of the products formed is large with respect to the amount of enzyme. The author has shown that the same experiments may be performed with pepsin.[3] In the case of these two enzymes, at least, therefore, there is direct experimental evidence for Arrhenius' explanation.

Effect of Constant Quantity of Inhibitor on Increasing Amounts of Trypsin.

In all the foregoing experiments the concentration of trypsin has been the same in any one series of experiments and the concentration of inhibitor varied. If the mechanism proposed is correct it should be possible to predict equally well the result of an experiment in which the concentration of inhibitor was kept constant and the amount of trypsin varied. That this is the case is shown in Fig. 2. The calculated results for this experiment were obtained by using the

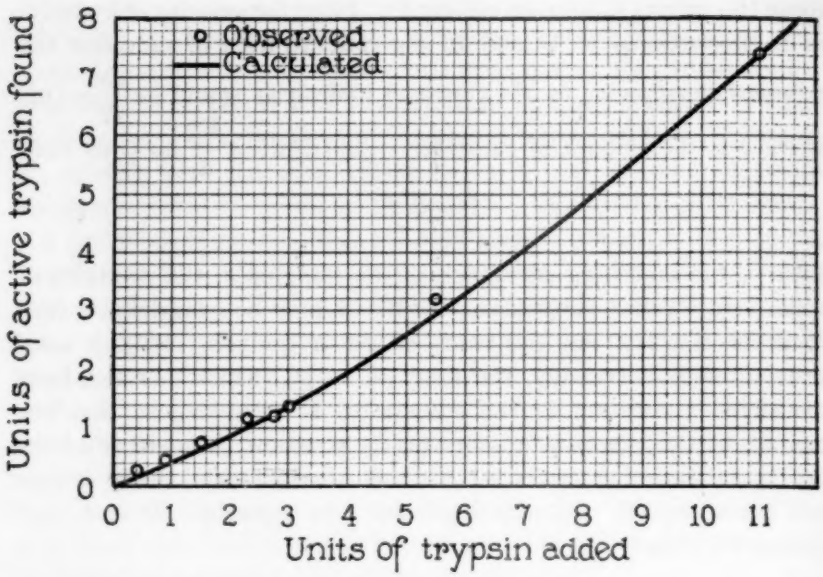

FIG. 2. The influence of the total amount of trypsin on the inactivation caused by 5 units inhibitor. Increased amounts of trypsin were added to series of tubes each containing 25 cc. gelatin solution, and 5 units inhibitor. Duplicate series run at the same time and under the same conditions, but without inhibitor.

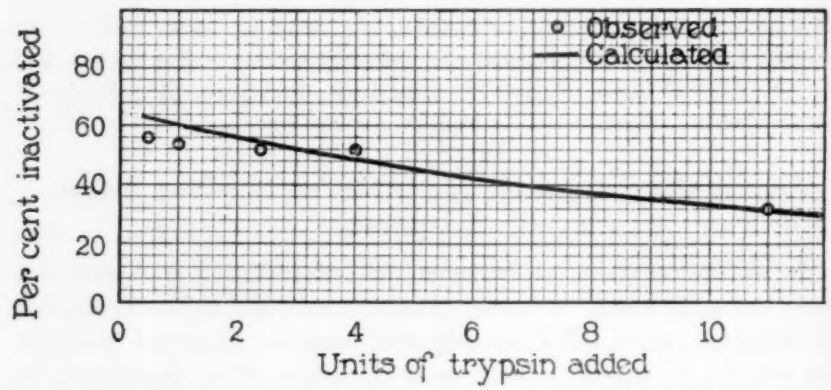

FIG. 3. The influence of the total amount of trypsin present on the percentage retardation caused by 5 units of inhibitor.

values for the constants obtained from the preceding experiments and were worked out before the experiment itself was done. The figure shows that in this case also the experimental and calculated results agree within the limit of experimental error. In Fig. 3 the percentage inactivation of the various amounts of trypsin by the five units of inhibitor have been plotted. As would be expected the smaller the amount of trypsin the greater the percentage inactivation although the absolute amount of trypsin inactivated is less.

It has been shown above that the law of mass action predicts quantitatively the results of the experiments when either the trypsin or the inhibitor concentration is varied. It is possible to vary conditions in another way by keeping the relative amount of trypsin and inhibitor the same and varying the dilution (*i.e.*, the value of v). The calculated and observed results of such an experiment are given in Fig. 4 (Curve *B*). The experiment was performed by mixing the trypsin and inhibitor solution and then adding the noted cubic centimeters of this mixture to 25 cc. of gelatin. It will be seen that in this case also the predicted results are in close agreement with the experiment. In this case the rate of hydrolysis decreases more slowly than the total amount of trypsin taken. This is the result of the fact that as the dilution is increased the trypsin inhibitor compound dissociates and so liberates more active trypsin, so that the concentration of active trypsin does not decrease directly as the total trypsin. Exactly the same curve would be obtained for the rate of hydrolysis by hydrogen ions furnished by a weak acid if the total concentration of acid were plotted against the rate of hydrolysis. In Curve *C* in Fig. 4 the result of an experiment is given in which the concentration of trypsin is varied but the concentration of inhibitor is kept constant. This is a similar experiment to that described in Fig. 2. In this case the rate of hydrolysis decreases more rapidly than the concentration of the trypsin. This is the result of the fact shown in Fig. 3 that the percentage retardation of the action of trypsin with a constant concentration of inhibitor is the greater, the smaller the total amount of trypsin. Curve *A* in Fig. 4 is the dilution-activity curve for "pure" trypsin and gelatin. In this case the velocity is nearly directly proportional to the amount of trypsin taken. It is clear from these curves that unless care is taken to purify the

enzyme and protein solution used, activity-concentration curves may be found to be either convex or concave or a straight line. This probably accounts for the discrepancies in the literature in regard to this point. If the enzyme solution contained products of protein digestion, as is very likely to be the case, the rate of hydrolysis would not increase as rapidly as the enzyme concentration. If the protein solution was already partially hydrolyzed or contained some inhibiting substance, the velocity of hydrolysis would increase more rapidly than the enzyme concentration.

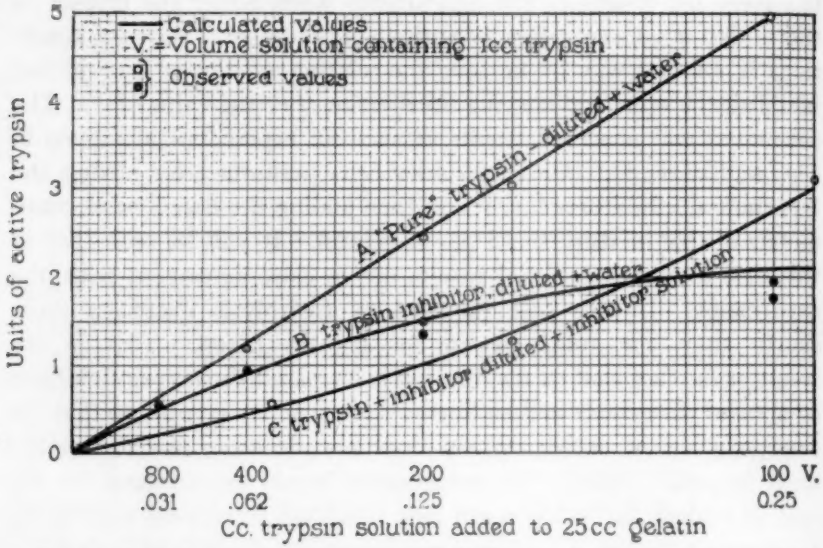

FIG. 4. The influence of the presence of inhibitor on the concentration-activity curve of trypsin. Curve *A*, "pure" trypsin diluted with water. Curve *B*, mixture of trypsin and inhibitor diluted with water. The ratio of trypsin to inhibitor is therefore constant. Curve *C*, mixture of trypsin and inhibitor diluted with a solution of inhibitor of the same concentration as was present in the trypsin solution. The concentration of inhibitor is therefore constant in this experiment.

Effect of the Hydrogen Ion Concentration.

In all the foregoing experiments the hydrogen ion concentration was kept constant at a pH of 6.3. It seemed of interest to determine what effect a variation in this factor would have on the retarding

action of the inhibiting solution. This experiment is difficult to perform since the conductivity method cannot be used and the pH can only be kept constant at ranges other than pH 6.3 by the use of high concentration of buffers which interfere with the formol titration. The results of such an experiment are given in Table V. The pH of the solutions was adjusted by making them all M/10 with respect to sodium carbonate and then titrating back to the desired pH with 0.1 N HCl. The course of the hydrolysis was followed by a slight modification of the formol titration already described.[6] No marked effect of the pH could be noted. Several other experiments were

TABLE V.

Effect of the Hydrogen Ion Concentration on the Inactivation of Trypsin by Inhibitor Solution.

A. Gelatin, 5 per cent, containing 0.2 N Na_2CO_3. *B*. Gelatin, 5 per cent, containing 0.2 N Na_2CO_3 and 4 per cent inhibitor solution. 50 cc. samples titrated to pH noted, and noted cc. trypsin added. Samples placed at 33°C. and formal titration run on 5 cc. at intervals.

pH at beginning pH at end	6.2 6.2	6.2 6.2	8.0 7.5	8.3 7.6	8.8 7.6	8.8 8.0	10.2 9.8	10.1 9.7
Cc. inhibitor per 50 cc.	0	2	0	2	0	2	0	2
Time in hours to cause increase of 1.0 cc. in formol titration	0.80	1.05	0.60	0.90	0.55	0.70	0.80	1.00
Per cent retardation	20		30		20		20	

made all of which gave results approximately the same as those given. No differences in the degree of retardation due to the pH were noted that could be definitely put outside the experimental error. This result was corroborated by the effect of the inhibiting solution on the rate of destruction of trypsin.[2] Here also no differences in the effect between pH 6 and 10 could be noted. This result seems to show that the "active" concentration of the trypsin and of the inhibiting substance is not markedly effected by the pH (between 6 and 10).

[6] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 595.

Does Trypsin Form a Compound with Gelatin?

The hypothesis which has been found to account quantitatively for the experiments described in this paper contains the assumption that the velocity of hydrolysis is proportional to the concentration of *free* enzyme. That is, the enzyme would enter into the formula for the kinetics of the reaction just as would the hydrogen ion concentration in the case of a reaction catalyzed by hydrogen ions or as one of the reactants in any chemical reaction and the time presumably consists of the time required for a molecule of enzyme to come in contact with a molecule of gelatin. The idea was proposed by Brown[7] and has since been elaborated by various authors that enzymes combine with the substances which are hydrolyzed by them and that the velocity of the reaction depends on the speed of decomposition of this compound, while the time for the compound to form is negligible. If this were true in the case of trypsin it is clear that the mechanism for the equilibrium between trypsin and the inhibiting substance which has been found to agree with the experiments could not be verified since it would be necessary to allow for the amount of trypsin combined with the gelatin. If this mechanism is correct therefore it seems necessary to conclude that the amount of trypsin combined with the gelatin at any one time is negligibly small and that the limiting time element is really the time required for the compound to form as is the case in other chemical reactions. The same reasoning evidently applies to pepsin hydrolysis and to invertase (Euler[4]), since in both these cases it has been found that the equilibrium between the enzyme and an inhibiting substance can be quantitatively accounted for by the assumption that the rate of hydrolysis is proportional to the *free* enzyme. This question will be discussed in another paper.

Can the Results be Accounted for by the Adsorption Formula?

The adsorption formula as given by Freundlich[8] and as usually used is written

[7] Brown, A. J., *J. Chem. Soc.*, 1902, lxxxi, 373.

[8] Freundlich, H., Kapillarchemie: Eine Darstellung der Chemie der Kolloide und verwandter Gebiete, Leipsic, 1909.

$$\frac{x}{C\frac{1}{n}} = k \cdot m$$

where x is the amount adsorbed, C is the concentration of the substance remaining in the solution, and m is the amount of adsorbent (or the area of the adsorbing surface). k and n are constants. In the present experiments it cannot well be assumed that the enzyme is adsorbed by the inhibiting substance since there is no evidence that this is in other than true solution. It would be necessary to suppose therefore that the inhibiting substance is adsorbed by the enzyme. The amount of enzyme would therefore be represented by m in the above formula and there would be no way to determine how much was combined and how much was free since there is no term in the equation to represent the amount of the adsorbent (in this case the enzyme) which is combined.

Hedin[9] has found that trypsin is adsorbed by charcoal and that the equilibrium in this case is that demanded by the adsorption formula. Hedin's experiments are, however, not contradictory to those described in this paper since it is quite possible that trypsin may be adsorbed by charcoal and yet react with other substances according to the law of mass action. This is known to be the case with acetic acid and many other substances.

SUMMARY.

1. A study has been made of the equilibrium existing between trypsin and the substances formed in the digestion of proteins which inhibit its action.

2. This substance could not be obtained by the hydrolysis of the proteins by acid or alkali. It is dialyzable.

3. The equilibrium between this substance (inhibitor) and trypsin is found to agree with the equation,

$$\text{trypsin} + \text{inhibitor} \rightleftharpoons \text{trypsin-inhibitor}$$

The equilibrium is reached instantaneously and is independent of the substrate concentration. If it be further assumed that the rate of

[9] Hedin, S., *Biochem. J.*, 1906, i, 484; *Z. physiol. Chem.*, 1906–07, l, 497.

hydrolysis is proportional to the concentration of the free trypsin and that the equilibrium conforms to the law of mass action, it is possible to calculate the experimental results by the application of the law of mass action.

4. The equilibrium has been studied by varying (*a*) the concentration of the inhibiting substance, (*b*) the concentration of trypsin, (*c*) the concentration of gelatin, and (*d*) the concentration of trypsin and inhibitor (the relative concentration of the two remaining the same). In all cases the results agree quantitatively with those predicted by the law of mass action.

5. It was found that the percentage retarding effect of the inhibiting substance on the rate of hydrolysis is independent of the hydrogen ion concentration between pH 6.3 and 10.0.

6. The fact that the experimental results agree with the mechanism outlined under 3, is contrary to the assumption that any appreciable amount of trypsin is combined with the gelatin at any one time; *i.e.*, the velocity of the hydrolysis must depend on the time required for such a compound to form rather than for it to decompose.

7. The experiments may be considered as experimental proof of the validity of Arrhenius' explanation of Schütz's rule as applied to trypsin digestion.

8. Inactivated trypsin does not enter into the equilibrium.

Many of the experiments described in this paper were carried out by Mr. Frank Johnston.

THE INACTIVATION OF TRYPSIN.

III. Spontaneous Inactivation.

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In addition to the inactivation of trypsin caused by its combination with some of the products of the hydrolysis, which has been discussed in the first part of this series, trypsin undergoes a second or spontaneous inactivation. This inactivation is independent of the action of the enzyme, irreversible and distinct from the reversible retardation of the action of the enzyme by the products formed during the reaction. Tammann[1] clearly recognized this complicating factor and attempted to correct for it, and there has since been considerable discussion as to the nature and course of this reaction. The rate of destruction of a number of enzymes has been studied, especially by Madsen and Walbum[2] who found in general that the reaction was monomolecular.

The inactivation of trypsin was studied by Vernon[3] who found that the reaction was not monomolecular but became progressively slower than the rate predicted by the monomolecular formula. He concluded therefore that the solution must contain a number of different forms of the enzyme some of which were more stable than others. He also found that, as is generally the case, the purity of the solution had a marked influence on the rate of decomposition. As will be seen from the experiments in this paper it is this factor which causes the divergence from the monomolecular formula so that it is unnecessary to assume the existence of a series of enzymes differing in their degree of stability.

[1] Tammann, G., *Z. physik. Chem.*, 1889, iii, 25.

[2] See Arrhenius, S., Immunochemie, Leipsic, 1907.

[3] Vernon, H. M., *J. Physiol.*, 1904, xxx, 330.

Methods.

The methods used in the present experiments were the same as those described above. The amount of active trypsin present was determined by measuring the time required for 1 cc. of the solution to cause a small amount of hydrolysis of a gelatin solution at 33° and a pH of 6.2 (in one experiment the pH was 10). The hydrolysis was followed either by the formol titration or the change in conductivity.

Influence of the containing vessels.—Since the formula for a monomolecular reaction is the same as that for diffusion it is necessary to know whether the enzyme is really being destroyed or simply diffusing to the walls of the containing vessel. In the latter case the rate of disappearance of the enzyme will depend on the size and character of the containing vessel, while if the process is chemical it will probably be independent of these factors. In order to test this point trypsin solutions (prepared as described in the preceding paper) were placed in various vessels and kept at 22° for 17 hours. The activity of the solution was tested before and after this interval. The results of this experiment are shown in Table I. It is evident that the destruction of the enzyme is independent of the container and is therefore probably not a diffusion process. The same conclusion is indicated by the temperature coefficient.

TABLE I.

Influence of Containing Vessel on Decomposition.

10 cc. dialyzed trypsin placed in vessel noted, and left at 20°C., pH 6.2. trypsin determined in 1 cc. after interval noted.

Time at 22°.	Trypsin per cc. after time noted at 22°C.			
	Containing vessel.			
	Platinum 5 cc.	Quartz 10 cc.	Paraffin 10 cc.	Glass 50 cc.
hrs.				
0	1.16	1.19	1.10	1.20
17	0.64	0.60	0.68	0.66

Influence of the Purity of the Solution on the Course of the Reaction.

Fig. 1 contains the results of a series of experiments on the rate of inactivation of various trypsin solutions at 38° and pH 6.2. The amount of active trypsin remaining in solution at any time has been plotted as the logarithm so that if the reaction were monomolecular the resulting curve would be a straight line. As the figure shows this is true in the case of the dialyzed trypsin. This particular solution had been dialyzed under pressure for 18 hours at 6°C., filtered and redialysed. The constant found is 0.005 (time in minutes and common logs). This experiment could not be repeated with certainty but in general, the more carefully the solution was purified the more nearly the reaction was found to be monomolecular. The figure also shows that undialyzed trypsin solutions and those to which gelatin had been added are apparently inactivated at first more rapidly than the pure solutions and then much more slowly. On the other hand, solutions containing inactivated trypsin or substances which had been found to interfere with the action of the enzyme, are much more stable, and if a large amount of these substances are present amount of decomposition is too small to determine in the interval of time chosen. The addition of glycine is without effect.

It has been shown in the previous paper that the products formed by the action of trypsin on proteins form a compound with the trypsin that is inactive. The simplest explanation for the present experiments would be to assume that exactly the same mechanism is at work here and that the compound, trypsin-inhibitor, is stable as well as inactive. It was found that the experiments referred to were in quantitative agreement with the hypothesis that the trypsin and inhibiting substance unite to form a compound according to the equation

$$\text{trypsin} + \text{inhibitor} \rightleftharpoons \text{trypsin-inhibitor}$$

and further that this equilibrium conformed to the law of mass action; *i.e.*

$$\frac{\text{Concentration of free trypsin} \times \text{Concentration of free inhibitor}}{\text{Concentration of trypsin-inhibitor}} = \text{a constant}$$

It follows from this equation that the amount of free trypsin present in a solution containing a given amount of inhibitor is a function of the dilution. The more concentrated the solution the more trypsin will be combined. Since the determination of the amount of free trypsin was made by adding 1 cc. of the trypsin to 25 cc. of gelatin it is possible for most of the trypsin to be active (uncombined) under these conditions but nearly all combined (inactive and stable) in the undiluted trypsin solution.

If the trypsin solution at the beginning of the experiment contains undigested protein it is evident that the concentration of the inhibiting substance, which is found by the action of trypsin on the protein, will increase during the experiment, and that the observed result will be the combination of two effects: first, reversible inactivation of the trypsin due to the presence of the inhibiting substance, and second, irreversible destruction of the free trypsin. The combination of these two factors will give a curve which, compared to the curve for the pure trypsin solution, will drop too rapidly at first, due to the increasing concentration of inhibiting substance and will then decrease too slowly owing to the fact that the trypsin is nearly all combined and therefore stable. This is the result shown in Fig. 1. This explanation may be verified by diluting the solution sufficiently before determining its activity. Since the inactivation due to the inhibiting substance is reversible and depends on the concentration of the solution whereas the spontaneous inactivation is irreversible, the two effects may be separated in this way and the resulting value alone will be a measure of the irreversible spontaneous decompositions. The result of an experiment performed in this way is shown in Fig. 2. The curve for the formol titration of the trypsin solution alone is also given. The figure shows that when the trypsin solution is sufficiently diluted before the determination is made, the initial rapid drop disappears and also that the time during which this drop is noticeable in the less diluted solution corresponds to the time during which the formol titration is increasing.

There remains to be explained the subsequent retardation of the inactivation in solutions containing protective substances. It follows from the law of mass action which, as has been shown, correctly expresses the equilibrium, that the smaller the amount of trypsin

present the greater the percentage of combined and therefore stable trypsin. In a solution, therefore, originally containing trypsin and inhibiting (protective) substances, the percentage of the trypsin that

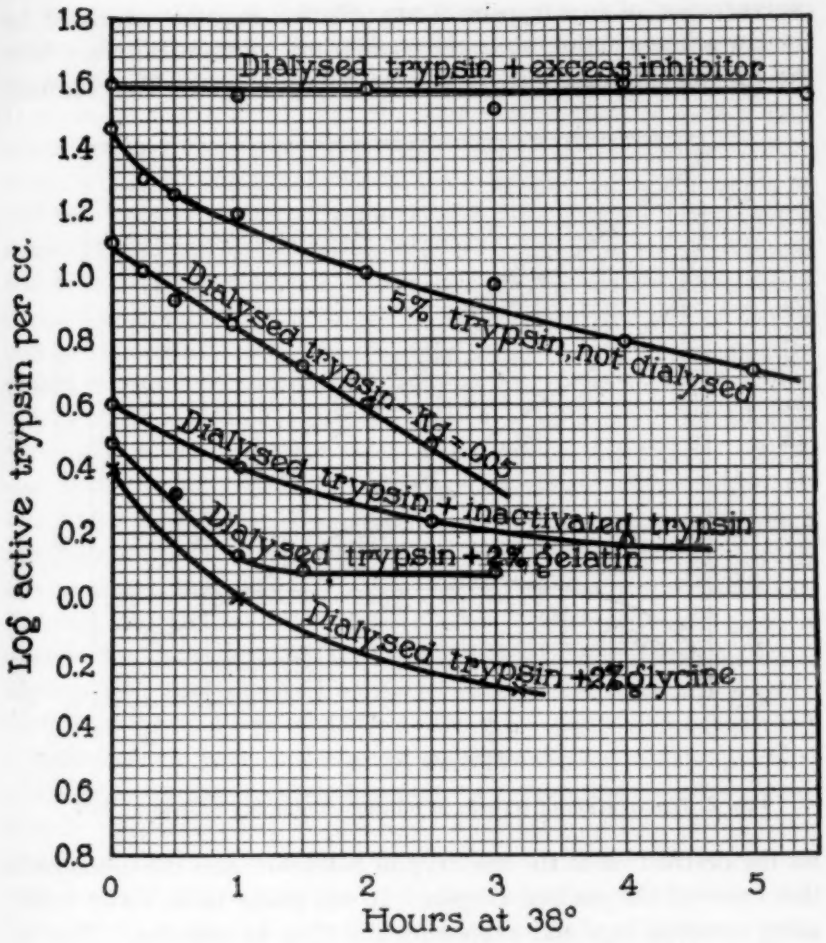

FIG. 1. Inactivation of various trypsin solutions at 38°C.

is free is constantly decreasing. Since it is this quantity that determines the rate of inactivation, the rate of inactivation will also constantly decrease and the resulting decomposition curve will fall more

slowly than demanded by the monomolecular formula. As has been stated this is the experimental result.

The above explanation may be tested quantitatively as follows. If the total concentration of trypsin and inhibitor be known the concentration of free trypsin at any dilution may be calculated by the law of mass action, since the equilibrium constant is known from the experiments described in the preceding paper. The constant

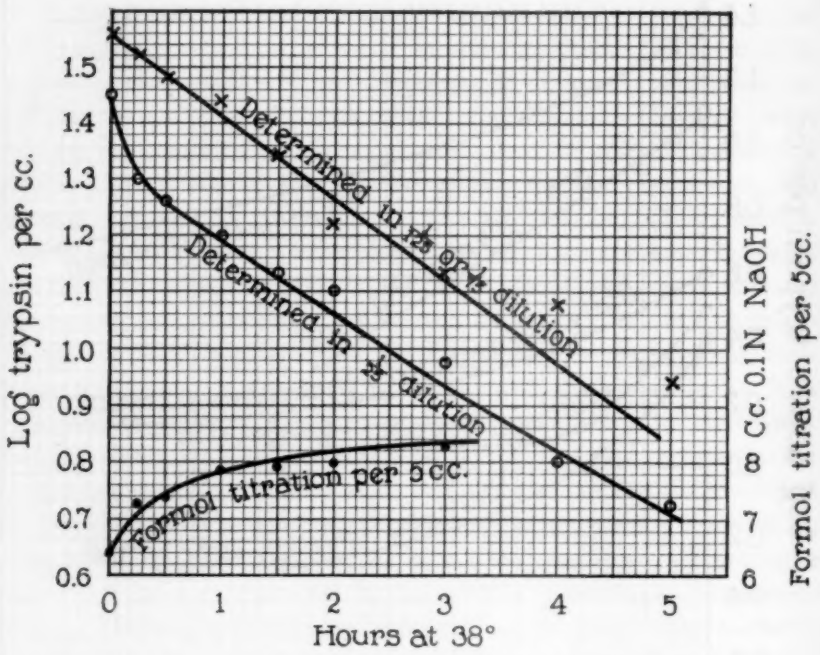

FIG. 2. Inactivation of trypsin in solution containing protein at 38°C.

for the destruction of the free trypsin is known from the decomposition curve of the purified trypsin. It was found to be about 0.005, using common logs and expressing the time in minutes. That is, very nearly half the amount of trypsin present is destroyed in 1 hour at 38° and a pH of 6.2. If a known amount of inhibitor is added to a known amount of trypsin, therefore, it is possible to calculate concentration of free trypsin in this solution. Since the value of *Kd* (the rate of decomposition) is known, the percentage of this free

trypsin that will be inactivated in any given interval of time can be approximated. The total amount of trypsin remaining in solution can then be found by difference and the amount of this which will be free and active at the concentration used in the determination, and in the presence of the known concentration of inhibitor can be calculated. This value should agree with that found by experiment. It must be remembered that the above calculation is only a first approximation since it contains two assumptions that are not strictly correct. (1) That the free trypsin is inactivated at the same rate as the same concentration of pure trypsin. As a matter of fact the amount of trypsin destroyed under the conditions of the experiment will be slightly greater than the quantity which would be destroyed if there were no combined stable trypsin present, since some of this will be dissociated as the free trypsin is destroyed and the amount of free trypsin and therefore the amount destroyed increased in this way. If the experiment is limited to the first part of the reaction (as was done) this difference is within the experimental error. (2) It was assumed that the only inhibiting (protective) substance present was added as the inhibiting solution. The decomposition curve of the trypsin solutions alone, however, show that in general there is always some protective substance present in the trypsin solution. The fact that the addition of inactivated trypsin renders the enzyme more stable indicates also that the solution contains some protective substances. This effect can hardly be ascribed to the inactive trypsin itself since it was shown in the first part of this paper that inactivated trypsin took no part in the reaction. The neglect of this quantity tends to make the calculated amount of trypsin destroyed too low. This is the result that is obtained. (If this quantity of inhibitor present in the trypsin solution is taken into account, the calculated and observed values may be made identical, but since there is no independent method of determining the value to be used, the process really consists in adding another arbitrary constant to the formula and so does not add much to the validity of the proof.)

The result of an experiment calculated and carried out as described above is shown in Table II. The agreement is as good as could be expected in view of the many sources of experimental error and of the fact that the calculation involves the extrapolation of the equilib-

rium equation over a range of 2,500 per cent dilution, since the values for the constants were determined in a solution which had been diluted 25 times and which in addition contained gelatin, whereas in the calculation the same formula was applied to a solution which had not been diluted at all and which contained no gelatin. This experiment appears to furnish strong confirmation of the validity

TABLE II.

Concentration of Inhibitor and Rate of Inactivation of Trypsin at 38°C.

10 cc. dialyzed trypsin and noted units inhibitor placed at 38°C. 1 cc. removed after intervals noted and added to 25 cc. of 2 per cent gelatin pH 6.2, specific conductivity 2.2×10^{-3}, at 33°C. and rate of hydrolysis followed; this gives the units of free trypsin per cc. solution when diluted 1:26. $K = 0.1$. Kd (decomposition trypsin) = 0.005.

Experiment No.	Inhibitor per cc. trypsin solution.	Free trypsin per cc. solution diluted 1:26 after hours at 38°C.					
		0 hours.		0.6 hours.		1.0 hours.	
		Observed.	Calculated.	Observed.	Calculated.	Observed.	Calculated.
	units	*units*	*units*	*units*	*units*	*units*	*units*
1	0	3.45	(3.45)	2.6	2.5		
1	0.17	3.40	3.35	3.0	2.3		
1	0.50	3.0	3.2	2.9	2.2		
1	1.7	2.3	2.6	2.2	2.15		
		2.5					
2	0	2.5	(2.5)			1.1	1.25
2	1.7	1.9	1.8			1.7	1.5
		2.0					
3	0	3.3	(3.3)			1.65	1.65
3	1.8	2.7	2.75			2.2	1.8

of the mechanism proposed for the reaction between the trypsin and inhibitor. It also shows that the equilibrium is not effected to any extent by the gelatin. The conclusion seems unavoidable that little or no trypsin is combined with the gelatin. The fact that gelatin has no protective influence on the trypsin also points to the same conclusion.

The Influence of the Manner in which the Inhibiting Solution is Added.

It was found in the study of the effect of the inhibiting solution on the rate of digestion by trypsin that the order in which the solutions were mixed and the time of standing was without effect on the result provided the control solution of pure trypsin did not alter during the experiment. If, however, the experiment is made at 38° where the control solution is rapidly destroyed the results are very different. The results of such an experiment are shown in Fig. 3. It will be seen that the solution to which all of the inhibitor had been added at the beginning of the experiment is much more active at the end than the one to which the inhibitor was added at intervals. This is evidently very similar to the Danysz phenomenon in immunology. In the present experiment the result is more marked if a relatively small quantity of the inhibitor (= antitoxin) is added at the beginning of the experiment whereas in the Danysz phenomenon it is necessary to add an excess. This is due to the fact that in the present experiment it is the free trypsin (= toxin) that is irreversibly changed during the experiment while in the Danysz experiment it is apparently the free antitoxin (= inhibitor) which is altered.[2]

The Decomposition of the Trypsin-Inhibitor Compound.

The rate of destruction of this compound at 38° is so slow in comparison to that of the free trypsin that it may be neglected. At 62°, however, it decomposes quite rapidly and follows the course of a monomolecular reaction (as was to be expected) provided an excess of inhibitor has been added so that practically all the trypsin is combined in the undiluted solution. The result of an experiment is given in Fig. 4. Irregular results were obtained in some cases owing to the formation of a precipitate presumably of coagulated protein. In such cases the amount of active trypsin remaining in solution shows a sudden drop at the time of formation of the precipitate.

Influence of the pH on the Rate of Decomposition.

Fig. 5 contains the result of an experiment in which the trypsin solutions were adjusted to various pH by the addition of HCl or NaOH before being placed at 38° for 1 hour. The activity of the

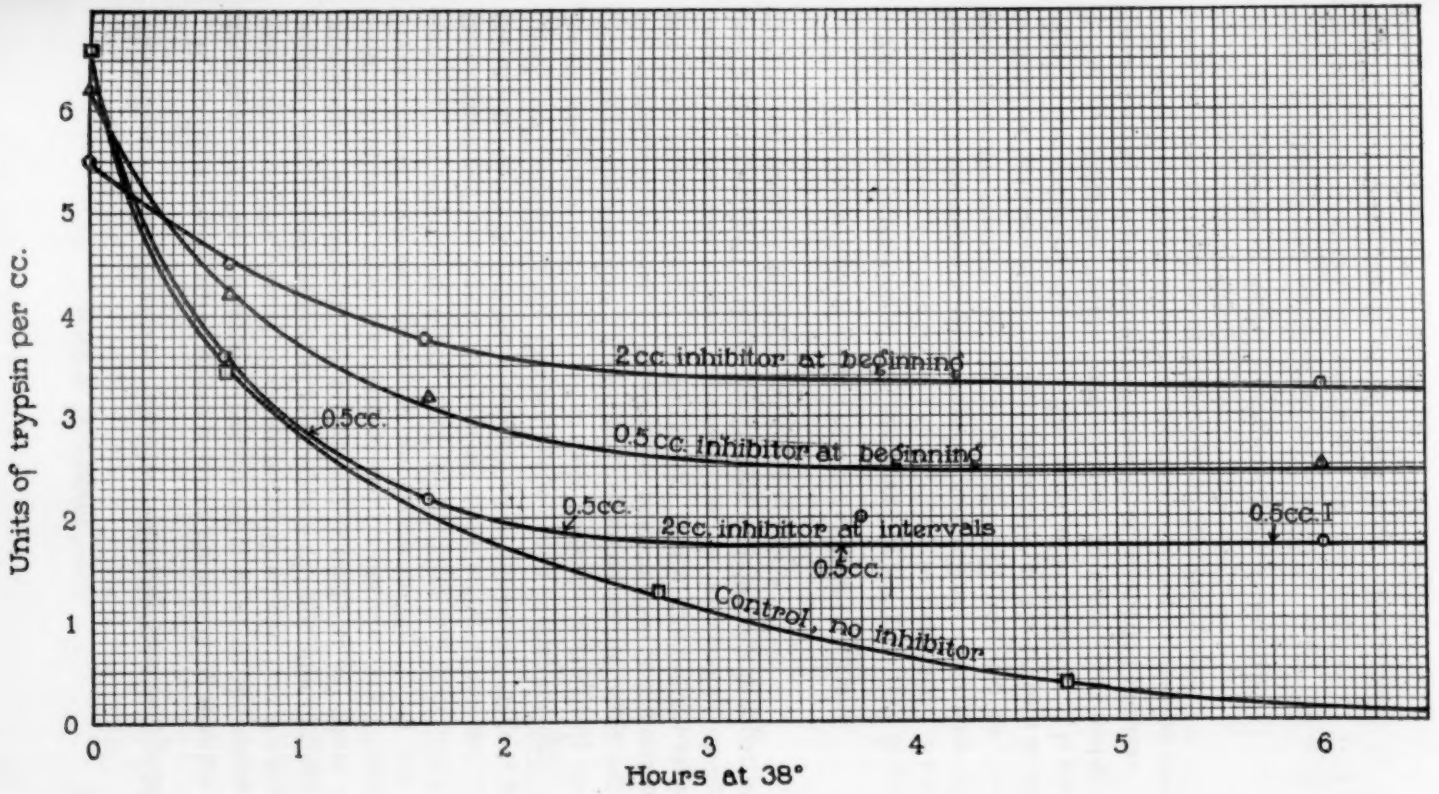

FIG. 3. Effect of time of adding inhibitor on decomposition of trypsin at 38°C.

solutions was determined by adding 1 cc. to 50 cc. of 5 per cent gelatin containing 0.2 M Na_2CO_3 (pH = 10) and the rate of hydrolysis followed by the formol titration. Trypsin is evidently most stable at a pH of 5. The rate of decomposition increases quite rapidly if the solution is either more or less acid.[4] It differs in this respect from pepsin which is stable over quite a wide range and resembles the invertase studied by Hudson.[5] The rapid increase in the rate of destruction

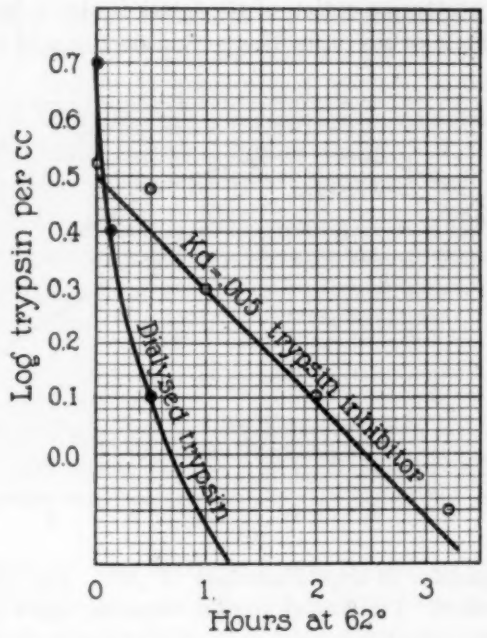

FIG. 4. Inactivation of trypsin and trypsin-inhibitor compound at 62°C.

with increasing alkalinity makes it evident that this behavior must enter to a large extent in deciding the optimum pH for the action of the enzyme. It was not found possible to determine quantitatively the effect of the pH owing to the fact that the reactions are rarely strictly monomolecular and it is therefore difficult to find a value

[4] This agrees with the experiments of Ringer (Ringer, W. E., *Z. Physiol. Chem.*, 1921, cvi, 107).

[5] Hudson, C. S., and Paine, H. S., *J. Am. Chem. Soc.*, 1910, xxxii, 774.

which correctly expresses the rate of decomposition. The time required for a certain amount to be destroyed could not be determined owing to experimental difficulties.

Influence of the pH on the Protective Effect of the Inhibiting Substance.

A comparison of the curves (Fig. 5) for the dialyzed trypsin solution and the solution to which inhibiting substance had been added shows that the protective action of the latter is also a function of the pH. There is little or no protective action on the acid side of pH 5.

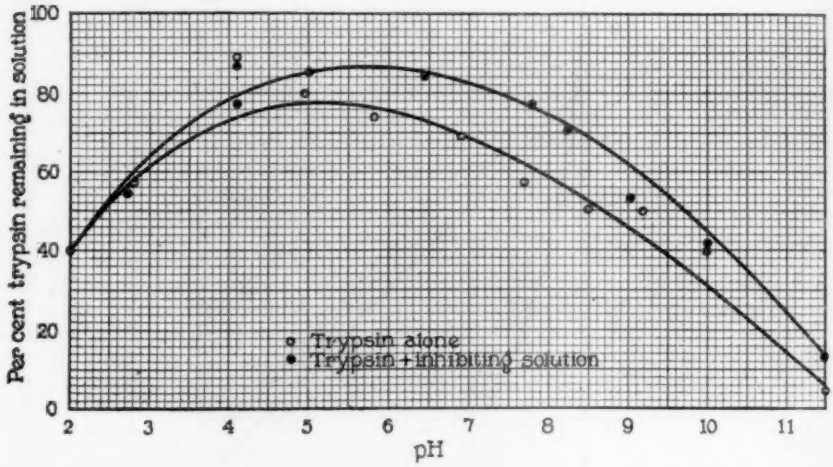

FIG. 5. Decomposition of trypsin solutions at 38°C. and different hydrogen ion concentrations. Per cent of trypsin remaining active after 0.5 hours at 38°C.

As the solution becomes more alkaline the protective action increases and then decreases slightly although the experiments are hardly accurate enough to be certain of this second decrease. If the hypothesis which has been used to account for experiments so far is correct this behavior evidently means that on the acid side of pH 5, trypsin does not combine with the inhibiting compound and that the combination has a maximum somewhere near a pH of 8 to 9.

This experiment confirms those described in the preceding paper in which it was found that the retarding influence of the inhibiting solu-

tion was not markedly effected by variations in the pH of the solution in the range of pH 6 to 10.

The mechanism which has been found to agree with the experiment described in this paper will also account for a peculiar fact which has been frequently observed in the study of the destruction of enzyme; namely, that the rate of decomposition at any one concentration will be strictly monomolecular, but that the rate becomes increasingly greater the more dilute the solution, instead of being independent of the concentration as is demanded by the monomolecular formula. If, as has been shown to be true for trypsin, the rate of decomposition depends on the amount of uncombined enzyme, it follows that the more dilute the solution the more rapidly the enzyme will become inactivated since the enzyme-inhibitor compound dissociates with increasing dilution. If, further, the inactivated enzyme reacts with the inhibitor to the same extent as does active enzyme (which was found to be the case with pepsin) then the rate of decomposition will be strictly monomolecular at any one concentration but will be the greater the more dilute the solution. This is the experimental result.

SUMMARY.

1. The rate of inactivation of purified trypsin solutions approximates closely that demanded by the monomolecular formula. The more carefully the solution is purified the closer the agreement with the formula.

2. The products formed by the action of trypsin on proteins renders the trypsin more stable. Gelatin and glycine have no effect.

3. The rate of inactivation of trypsin solutions containing these products does not follow the course of a monomolecular reaction but becomes progressively slower than the predicted rate.

4. The protective action of these substances is much greater if they are added all at once at the beginning of the experiment than if they are added at intervals.

These observations may be quantitatively accounted for by the hypothesis that a compound is formed between trypsin and the inhibiting substance which is stable as well as inactive, and that the rate of decomposition depends on the amount of uncombined trypsin present.

5. Trypsin is most stable at a pH of 5 and is rapidly destroyed in strongly acid or alkaline solution.

6. The protective effect of the inhibiting substances is small on the acid side of pH 5, increases from pH 5 to 7, and then remains approximately constant.

DIRECT AND INDIRECT DETERMINATIONS OF PERMEABILITY.

By W. J. V. OSTERHOUT.

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Lack of direct and satisfactory means of determining the penetration of substances into the living cell has greatly hampered the study of permeability. Various indirect methods have been resorted to, but their reliability must remain in question until a direct method can be found for testing them.

Investigators have sought to overcome this difficulty in various ways. Some have made analyses of tissues, but it is evident that these must include too much intercellular material to be satisfactory. Analysis of the solution in which the tissue lies, in order to determine what is absorbed, is open to the objection that substances collect on the surfaces of cells, as well as in the cell walls and in the spaces between them, so that it is impossible to say what actually penetrates the protoplasm. Others have sought to analyze the cell sap. Plant cells are most favorable for this purpose, since, as a rule, they contain vacuoles filled with sap. In general the method has been to crush the tissues and express the sap, but this procedure involves many possibilities of error.[1]

[1] Among these may be mentioned contamination of the cell sap by substances present in the cell walls or intercellular spaces and chemical reaction between the cell sap and the crushed protoplasm or the cell walls. The degree of pressure used in expressing has a marked influence on the concentration of the sap (*cf.*, Mameli, E., *Atti del r. inst. bot. de Pavia*, 1908, xii, 285; Dixon, H. H. and Atkins, W. R. G., *Sci. proc. roy. Dublin Soc.* N. S. 1913, xiii, 422; Gortner, R. A., Lawrence, J. V., and Harris, J. A., *Biochem. Bull.*, 1916, v, 139).

The investigation of blood and other body fluids is open to the objection that we do not know to what extent substances penetrate between the cells in reaching these fluids. In many cases penetration into these fluids seems to present very special features.

The taking up of dyes has been extensively investigated but this method is beset by many pitfalls[2] and the results hitherto obtained are confusing.

In some cases the penetration of acids and alkalies has been studied by means of organisms containing natural indicators, or by introducing indicators into the cell.[3] Use has also been made of the fact that the penetrating substance may cause a visible precipitate within the cell; this is especially the case with alkaloids.[4] Furthermore the absorption of Ca[5] has been detected by observing the formation of crystals of Ca oxalate within the cell. It is evident, however, that these methods have but limited application, and that in many cases they are open to the objection that the penetrating substance injures the cell.

The penetration of a substance may sometimes be demonstrated by observing its effect upon metabolism, but this method is inadequate from a quantitative standpoint. Some investigators contend that substances may produce effects on metabolism by their action at the surface, without actually penetrating the cell.

[2] To a great extent the coloration of the cell by a dye shows the extent to which the dye can combine with the substances within the cell rather than the rate at which the dye penetrates. Thus many cells contain substances which combine with methylene blue so that it becomes far more concentrated within the cell than in the external solution (Pfeffer, W., Physiology of plants, 2nd edition, Oxford, 1897, i, 96). Unless the cell has this power it often fails to appear colored even though it may contain the dye in the same concentration in which it exists outside. In such cases it may sometimes be detected by plasmolyzing the cell and thus concentrating the dye. A further complication is that a cell may appear to have taken the dye into its interior when in reality only the surface or the cell wall is stained. There are many other difficulties, which need not be discussed here, such as toxic action of the dye and changes in the dye (decolorization, etc.) as it enters the cell. A very serious objection to this method is that it does not give quantitative results. A review of the literature will be found in Höber, R., Physikalsche Chemie der Zelle und der Gewebe, 4th edition, Leipsic and Berlin, 1914.

[3] For the literature see Haas, A. R., *J. Biol. Chem.*, 1916, xxvii, 233; Crozier, W. J., *J. Biol. Chem.*, 1916, xxiv, 255; xxvi, 225; 1918, xxxiii, 463.

[4] For the literature see Czapek, F., Ueber eine Methode zur direkten Bestimmung der Oberflächenspannung der Plasmahaut von Pflanzenzellen, Jena, 1911.

[5] Osterhout, W. J. V., *Z. physik. Chem.*, 1910, lxx, 408.

It is evident that the most satisfactory method is to place the cell in a solution containing the substance whose penetration is to be investigated and, after a definite time of exposure, to obtain the cell sap without contamination and test it for the presence of this substance. Experiments of this sort have apparently not been carried out, though interesting results have been obtained by Meyer,[6] Hansen,[7] Wodehouse,[8] and Crozier[9] by comparing the cell sap of the marine alga *Valonia* (which can be obtained without contamination) with the sea water. Janse[10] found that filaments of *Spirogyra* which had been kept for a time in a solution of KNO_3 gave a test for NO_3 after being rinsed and caused to burst in a solution of diphenylamine. In this method there is serious risk of contamination by substances in and upon the cell wall (or between the cell wall and the protoplasm).

In order to avoid this difficulty the writer has employed the large, multinucleate cells of a species of *Nitella*, some of which reach a length of 6 inches and a diameter of a thirty-second of an inch.

Within the cell wall the protoplasm forms a thin layer in which the chlorophyll bodies are imbedded. Inside this layer is the large central vacuole filled with cell sap. It is possible to obtain the cell sap without contamination in various ways. The writer has made use of the following methods: The cells are placed for the desired length of time in a solution containing the substance whose penetration is to be tested. They are removed, washed in running tap water (followed in many cases by distilled water), and dried by means of filter paper. The cells are so large and turgid that this manipulation presents no difficulty. A cell is then placed on a piece of glass or filter paper and pierced with the point of a clean capillary tube (which has been drawn out to a fine tip). The cell sap is drawn up into the tube (by capillary action) quite free from protoplasm or chloroplasts.[11] Another method, which is preferable in many cases,

[6] Meyer, A., *Ber. deutsch. bot. Ges.*, 1891, ix, 77.

[7] Hansen, A., *Mitt. Zool. Stat. Neapel*, 1893, xi, 255.

[8] Wodehouse, R. P., *J. Biol. Chem.*, 1917, xxix, 453.

[9] Crozier, W. J., *J. Gen. Physiol.*, 1918–19, i, 581.

[10] Janse, J. M., *Versl. Meded. Kon. Akad. Wetensch. Afd. Natuurk.* (3), 1888, iv, 332.

[11] During the manipulation care should be taken to prevent the sap from running out of the cell and coming in contact with its outer surface.

is to suspend the cell by a pair of forceps attached to the upper end, cut off the lower end and bring it in contact with a glass slide, and then grasp the upper end gently with another pair of forceps which is slowly moved downwards while a slight pressure is maintained. The cell sap then flows out on to the glass slide. By uniting the drops from a number of cells it is possible to get a sufficient amount for qualitative chemical tests, and in many cases approximate quantitative results may be obtained.

Since in previous investigations the writer had employed indirect methods of testing permeability it was of considerable interest to compare such results with those obtained by direct tests of the cell sap. An investigation was therefore made in which the permeability of *Nitella* was tested by determinations of plasmolysis and of electrical conductivity as well as by the direct method. This may be illustrated by a series of experiments[12] with $NaNO_3$ and $Ca(NO_3)_2$.

Experiments on plasmolysis were carried out by placing the cells in a hypertonic solution and observing the time required to recover from plasmolysis (without removing the cells from the solution) on the assumption that the more rapid the recovery the more rapid is the penetration of the salt.

In these experiments the smaller cells near the tip of the plant were largely employed. They were observed in Syracuse watch-glasses, or placed on glass slides and covered with large cover-glasses the edges of which were sealed with vaseline. The experiments require continuous observation of individual cells, since (especially in unbalanced solutions) recovery is promptly followed in many cases by injury and false plasmolysis,[13] which may be mistaken for true plasmolysis.

It is evident that plasmolysis may be injurious to many cells even in a balanced solution;[14] while in an unbalanced solution there may be

[12] All the experiments were performed at about 19°C. All the solutions were approximately neutral.

[13] Osterhout, W. J. V., *Bot. Gaz.*, 1908, xlvi, 53; 1913, lv, 446; *Science*, 1911, xxxiv, 187.

[14] For this reason penetration may be more rapid than would otherwise be the case. In order to reduce toxicity pure salts should be used and the water should be distilled from quartz (or from glass which has been in use for some time), using cotton plugs in place of rubber or cork stoppers, and rejecting the first and last parts of the distillate.

the additional injury due to the toxic action of the salt. For this reason many cells which would recover if very slightly plasmolyzed may not do so if plasmolyzed more strongly, since recovery may require so much time that the process of injury gets the upper hand.

It was found that recovery was more rapid in $NaNO_3$ than in a balanced solution of $NaNO_3$ plus $Ca(NO_3)_2$ or in $Ca(NO_3)$ or $CaCl_2$ alone. Experiments with RbCl and CsCl, with and without the addition of $CaCl_2$, gave similar results. This indicates that in a solution of $NaNO_3$, NaCl, RbCl, or CsCl penetration is more rapid than in $Ca(NO_3)_2$, $CaCl_2$, or a balanced solution.

These results agree with those previously obtained by the writer in studying *Spirogyra*.[15]

The experiments on conductivity were carried out by means of an apparatus consisting of a block of paraffin (*P*, Fig. 1) in which are two depressions containing platinum electrodes, *E*, *E*, covered with platinum black. A connecting groove contains the cells, *N*, loosely packed together and covered by a glass plate, *G*. The cells are placed in the groove after it is filled with liquid and great care is taken to avoid air bubles. When a new solution is to be introduced it is poured into *T*; it then runs through the groove and escapes by the opening *O*; the irrigation is continued until the old solution is completly removed.

Before beginning the experiments the conductivity of the cell sap was tested[16] and found to be equivalent to that of sea water diluted with three parts of tap water (this will be referred to a 0.25 sea water). As the cells grew well in 0.20 sea water they were kept in this for some time before each experiment and then transferred to 0.25 sea water just before the experiment. The conductivity of the cell sap was then approximately the same as that of the external solution and the fact that the cells had a greater resistance than the solution must be ascribed to the greater resistance of the protoplasm or of the cellulose wall. When the cells were killed by agents which produce no alteration in the cell wall the resistance fell to approximately that of the solution. It is therefore evident that the increased resistance was due to the protoplasm.

[15] Osterhout, W. J. V., *Science*, 1911, xxxiv, 187.

[16] For this purpose a capillary tube was filled with sap and a platinum electrode was inserted into each end of the tube, care being taken to avoid air bubbles.

The conductivity was first determined in 0.25 sea water, this was then replaced by another solution of the same conductivity. The conductivity in the new solution was then determined at intervals.

In general it was found that in solutions in which the cells lived well, such as dilute sea water or a balanced solution of $NaNO_3$ plus $Ca(NO_3)_2$, the resistance did not fall. But in solutions in which injury occurred, such as $NaNO_3$, the resistance fell. In $Ca(NO_3)_2$ and in $CaCl_2$ there was a rise in resistance followed by a fall.

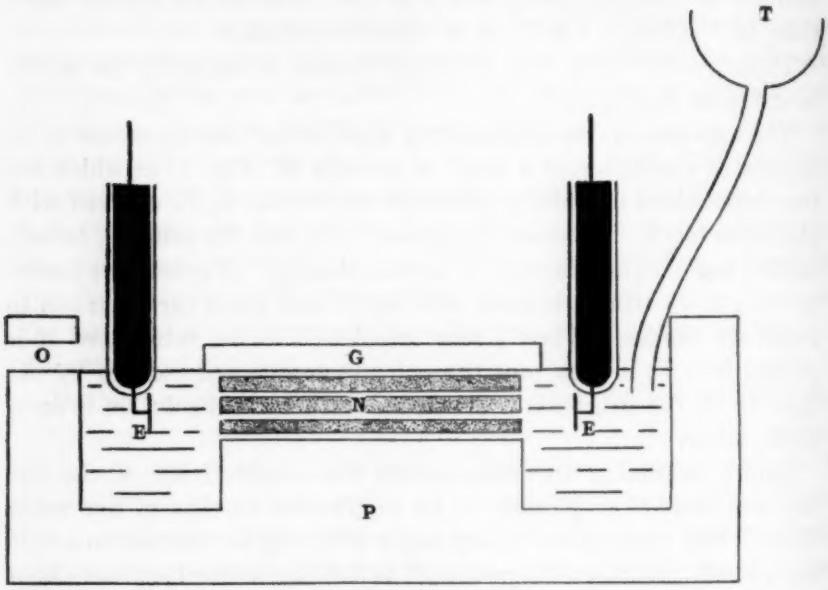

FIG. 1. Apparatus for measuring the conductivity of *Nitella*. The cells (*N*) are placed in a trough in a block of paraffin (*P*) and covered with a glass plate (*G*). The solution is poured in through the funnel (*T*) and runs out through the opening (*O*). At *E* and *E* are platinum electrodes.

These experiments agree with those made on *Laminaria* except that the rise observed in $Ca(NO_3)_2$ or in $CaCl_2$ was often smaller in the case of *Nitella*. This, however, is not unexpected, since the concentration of $CaCl_2$ was only one-fourth of that used in the experiments on *Laminaria*. It may be added that the rise observed in $CaCl_2$ differs greatly in different organisms. In *Rhodymenia*,[17]

[17] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 299.

for example, it is very small and temporary, even in 0.278 M $CaCl_2$ (which has the conductivity of sea water).

As was to be expected from experiments on other organisms[18] $La(NO_3)_3$ also produces a rise in resistance which is followed by a fall.

One fundamental principle is evident in the results of all the experiments, including those on plasmolysis and on electrical conductivity; namely, that in solutions which are injurious the resistance eventually falls, while in non-toxic solutions it remains practically unaltered. This has been interpreted as indicating that injury is accompanied by an increase of permeability, but the evidence for this view was obtained by indirect methods and the writer welcomed the opportunity to test it by the direct method of examining the cell sap.

The experiments on sap were confined to the determination of NO_3, since it was found that the cells normally give tests for Na and Ca. Since the method employed was not sensitive enough to detect NO_3 in the sap of the control cells under any circumstances, it is evident that if a test was obtained after exposure to a solution containing NO_3 it must have been due to penetration from without.

The sap was tested by placing it on a glass slide, adding a drop of nitron dissolved in 10 per cent acetic acid, and observing it under the microscope. If NO_3 is present it may be recognized by the formation of characteristic crystals.

Cells kept for 24 hours in 100 cc. $NaNO_3$ 0.05 M plus 10 cc. $Ca(NO_3)_2$ 0.05 M gave no test, which shows conclusively that the method is safe as far as contamination by NO_3 on the surface is concerned. After 48 hours a test was obtained. As the cells continued to live in this solution for 3 weeks (at which time the experiment was discontinued) and as they appeared normal in every way, it is evident that the penetration was not the result of injury.

It is probable that in 24 hours there was some penetration which was not revealed by the test. This, however, has no significance in the present investigation, the aim of which is not to determine the absolute amount of penetration but merely to compare the relative penetration in balanced and unbalanced solutions.

The results of such a comparison are very striking. After 3 hours in $NaNO_3$ 0.05 M a good test was obtained. The cells had lost some

[18] Osterhout, W. J. V., *Bot. Gaz.* 1915, lix, 464; *J. Gen. Physiol.* 1918-19, i, 299, 409.

of their turgidity; if left in the solution of $NaNO_3$ or if transferred to tap water they subsequently lost all their turgidity, indicating death. It is therefore evident that this rapid penetration was accompanied by injury.

It may be remarked that the turgidity of the cells is a good indication of their condition. It is easily tested by lifting them out of the solution; if in good condition they appear stiff, if injured they tend to collapse. It is, however, necessary to distinguish between loss of turgidity in isotonic or hypotonic solutions, which indicates injury, and a similar appearance in hypertonic solutions, which may indicate nothing of the sort. In the latter case the cell promptly recovers its turgidity when placed in tap water; in the former it does not.

Another criterion of injury is afforded by the appearance of the chlorophyll bodies. In the normal cell they are arranged in regular rows and are of a clear, transparent green color. When injury occurs they lose their regular arrangement and their color becomes more opaque.

In 0.05 M $Ca(NO_3)_2$ the cells live for a week or more. During the first few days, at least, penetration is not more rapid (perhaps is less so) than in a balanced solution.

Similar results were obtained with other salts, which will be described in a subsequent paper.

The outcome of these direct tests is therefore a confirmation of the results obtained by the indirect methods. We find that penetration in injurious solutions is relatively rapid as compared with penetration in non-toxic solutions. This corresponds to the fact that recovery from plasmolysis is more rapid in injurious solutions as well as to the fact that conductivity increases in such solutions.

In view of this we may conclude that determinations of electrical conductivity give reliable information regarding changes in permeability. Observations on recovery from plasmolysis, while giving similar results, are less satisfactory.

SUMMARY.

1. Methods are described for obtaining cell sap from *Nitella* without contamination.

2. Tests of the cell sap show that in a balanced solution of $NaNO_3$ plus $Ca(NO_3)_2$ there is a slow penetration of NO_3 and that the cell remains in a normal condition, but in pure $NaNO_3$ there is rapid penetration accompanied by injury.

3. Inasmuch as determinations of electrical conductivity give the same result it may be concluded that this method gives reliable information regarding permeability.

4. While observations on recovery from plasmolysis give similar results, the method is less satisfactory.

STUDIES ON BIOLUMINESCENCE.

XIV. The Specificity of Luciferin and Luciferase.

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INTRODUCTION.

In the living world are found more than thirty groups of organisms producing light, scattered, in the animal kingdom at least, through orders widely different in morphological characteristics. Physiologically, one order may contain individuals producing light in quite different ways, as the squids, some of which have light organs producing an external secretion of luminous material, while others possess light organs of internal combustion. Among the fish, also, are found various types of luminescence. Some forms emit light only as a result of stimulation while others luminesce continually day and night, and the intensity is quite independent of stimulation of any kind. In this respect these fish resemble the fungi and luminous bacteria which also emit a steady continuous light which is not varied on stimulation.

While it is impossible at the present time, because of lack of data, to classify accurately and logically the various types of luminescence found in living things, the accompanying list is an attempt in this direction, which will serve as a guide to the groups, whose particular characteristics of luminescence are discussed in this paper. Accordingly, relationship as well as physiological peculiarities of luminescence have been considered in making the group (Table I).

Two questions at once arise in connection with these data. The first has to do with the presence of the luciferin-luciferase reaction. As is evident from the above list, luciferin and luciferase, first dis-

TABLE I.

Groups of Luminous Organisms.

Group.	Genus studied.	Type of luminescence.	Luciferin and luciferase.
Bacteria.	*Microspira.* *Photobacterium.*	Intracellular and continuous.	Negative.
Fungi.	*Armillaria.* *Clytocybe.*	Intracellular and continuous.	?
Radiolaria.	*Thallassicola.*	Intracellular; on stimulation.	?
Dinoflagellates.	*Gonyaulax.* *Ceratium.*	Intracellular; on stimulation.	?
Cystoflagellates.	*Noctiluca.*	Intracellular: on stimulation.	Negative.
Sponges.	*Grantia.**	(?); on stimulation.	Negative.
Medusæ and hydroids.	*Æquorea.* *Mitrocoma.*	Extracellular (?); on stimulation.	Negative.
Pennatulids.	*Cavernularia.* *Pennatula.* *Ptilosarcus.*	Extracellular (?); on stimulation.	Negative.
Ctenophores.	*Bolina.*	Intracellular (?); on stimulation.	Negative.
Tomopterid worms.	*Tomopteris.*	Extracellular (?); on stimulation.	?
Syllid worms.	Odontosyllis.	Extracellular (?); on stimulation.	Positive.
Polynöed worms.	*Polynöe.*	Intracellular; on stimulation.	Negative ?
Chætopteroid worms.	*Chætopterus.*	Extracellular; on stimulation.	Negative
Earthworms.	*Microscolex.*	(?); on stimulation.	?
Ostracod crustacea.	*Cypridina.* *Pyrocypris,*	Extracellular; on stimulation.	Positive.
Copepod crustacea.	*Metridia.* *Pleuromma.*	Extracellular; on stimulation.	?
Schizopod crustacea.	*Meganyctiphanes.*	Intracellular; on stimulation.	Negative.
	Gnathophausia.	Extracellular; on stimulation.	?

* Luminescence doubtful.

TABLE I—*Continued.*

Group.	Genus studied.	Type of luminescence.	Luciferin and luciferase.
Decapod crustacea.	*Sergestes.*	Intracellular; on stimulation.	?
	Heterocarpus.	Extracellular; on stimulation.	?
Myriapods.	*Geophilus.*	Extracellular; on stimulation.	Negative ?
Springtails.	*Podura.*	Intracellular (?); on stimulation.	?
Beetles.	*Luciola.* *Pyrophorus.*	Intracellular; on stimulation.	Positive
Flies.	*Boletophila.*	Intracellular; on stimulation.	?
Brittle stars.	*Ophiacantha.*	Intracellular (?); on stimulation.	?
Lamellibranch mollusks.	*Pholas.*	Extracellular; on stimulation.	Positive.
Nudibranch mollusks.	*Phyllirrhöe.*	Intracellular (?); on stimulation.	?
Cephalopods.	*Watasenia.*	Intracellular; on stimulation.	Negative.
	Heteroteuthis.	Extracellular; on stimulation.	?
Balanoglossids.	*Glossobalanus.*	Extracellular; on stimulation.	Positive.
Salpids.	*Pyrosoma.*	Intracellular; on stimulation.	Negative.
Fish	*Photoblepharon.* *Anomalops.*	Intracellular; continuous.	Negative.
	Maurolicus. *Porichthys.*	Intracellular; on stimulation.	?
	Gonostoma.	Extracellular (?); on stimulation (?).	?

covered by Dubois[1] in *Pyrophorus*, an elaterid beetle, and *Pholas*, a mollusk can be demonstrated in several other groups of the animal kingdom but not in all. Why cannot these substances be demonstrated in all orders? It would seem that so fundamental a reaction should be universal; that is one question awaiting solution.

A second question concerns the specificity of luciferin and luciferase. Will the luciferase of one species produce light with the luciferin of another species, or genus, or group, and *vice versa?* We have here material for an interesting study of enzyme specificity and this is necessary, as we shall see, for a proper analysis of the first question, why luciferin and luciferase cannot be demonstrated in all groups of luminous animals.

The Luciferin-Luciferase Reaction.

The general methods for preparing luciferin and luciferase are very simple. Luciferin is made by adding hot water to the luminous organ of the animal or by quickly heating the luminescent extract of the luminous animal to temperatures which permanently quench the light, or to boiling. By this means the luciferase is destroyed on heating before the luciferin (which is not destroyed by heating) has been completely oxidized. Care must be taken to destroy the luciferase as quickly as possible, before it has had time to oxidize the luciferin. Hence the advantage of adding hot water suddenly to the luminous gland. Care must also be taken not to heat the luciferin to too high a temperature, or too long, as it may be destroyed under these conditions. Hence the advantage of heating a luminous extract to just the point where the light is permanently extinguished, and cooling quickly. Before deciding that luciferin cannot be demonstrated in an animal, these precautions have always been taken.

Luciferase is prepared by allowing a cold water extract of the luminous gland to stand until the luciferin has been completely oxidized. This oxidation can be accelerated by shaking the solution to aerate it well, or by gentle heating (not sufficient to destroy the luciferase), or by adding such substances as chloroform, saponin, or sodium glycocholate. These substances apparently act by liberating

[1] Dubois, R., *Compt. Rend. Soc. Biol.*, 1885, ii, 559; 1887, iv, 564.

luciferin bound (combined or absorbed) in some way in the solution, perhaps sometimes by causing cytolysis of still intact photogenic cells or by causing solution of photogenic granules or granulysis. Extracts of non-luminous animals sometimes contain substances acting like the above cytolytic agents. For these I have suggested the general term of photopheleins. Care has been taken to exclude such sources of error and misinterpretations in the studies described below.

It is obvious, from the method of preparation of luciferase, that, should there be just enough luciferase or less than enough luciferase to oxidize all the luciferin of a luminous gland, we could not obtain a solution of luciferase by the above method. Only if an excess of luciferase over luciferin exists can a solution of luciferase be obtained. It is possible, therefore, that this is the explanation of negative results for the presence of these bodies in certain groups of luminous organisms, a possibility that can be tested in part and that will be discussed below.

It is not to be supposed that inability to demonstrate luciferin and luciferase in a luminous form is always due to the same cause. Assuming that luciferin and luciferase really do occur in all luminous forms, it may be that they are present in such small amounts, compared with the bulk of non-luminous tissue necessarily included in extracting them, that no luminescence is visible. This might be the case in *Chætopterus*, an annelid worm, where luminous gland cells occur over the surface of the body. These cells cannot be removed individually and the most luminous regions of the worm must be extracted as a whole, involving a large mass of non-luminous material.

Again, either luciferin or luciferase or both may be very unstable in some forms, undergoing change before their presence in an extract may be tested.

Or, it is not impossible that the luciferase may occur in an endoenzyme condition, similar to the zymase of yeast or enzymes of bacteria, which render it impossible to extract except under special conditions and high pressures. I have concluded that such is the case in luminous bacteria and that this explains the absence of a luciferin-luciferase reaction in these forms.[2] It seems possible also, however,

[2] Harvey, E. N., *Am. J. Physiol.*, 1916, xli, 449.

that very little luminous material is present in these forms at any one time, but that it is manufactured continuously by the living bacterial cell.

But in addition to the forms which may contain only small amounts of luciferin and luciferase, or unstable luciferin and lucerferase, or luciferase in endoenzyme condition, there are at least two groups of animals which contain abundant luminous materials, whose light is long lasting, whose cells may be easily broken up, and in which the photogenic substances may be readily dried and give a bright light on again moistening. These are the medusæ, *Æquorea forskalea* and *Mitrocoma cellularia*, and the pennatulids, *Cavernularia haberi* and *Ptylosarcus* Sp.(?) especially the medusæ. *Æquorea and Mitrocoma*, found at Friday Harbor, Washington, contain many clumps of luminous cells about the rim of the umbrella at the base of the tentacles. Under the microscope masses of yellow material can be seen in the position from which the light comes, which probably are the photogenic cells. Gentle rubbing of the region liberates abundant luminous secretion which sticks to the fingers and which causes the sea water to luminesce quite brightly. The rim of the umbrella is easily cut away and this material, when squeezed through cheese cloth, gives a permanent bright luminous extract whose light lasts several hours. The animal itself luminesces only on stimulation.

There is, then, in these medusæ no lack of photogenic material. The material is readily extracted and stable, since the light lasts for several hours. Nevertheless, the luciferin and luciferase reaction cannot be obtained with these forms despite many attempts and care to guard against all sources of error.

What is the reason for this negative behavior? Is luminesence of jellyfish quite a different process from that in *Cypridina*, *Pholas*, or fireflies, which do give the luciferin-luciferase reaction? Or is the amount of luciferase in these forms just sufficient to oxidize the luciferin which is present and leave no excess in the extract? In *Cypridina* there is enough luciferase in one animal to oxidize the luciferin of 100 animals, but not an indefinite amount. *Cypridina* luciferase behaves as an enzyme but is not a perfect example of a catalyst which should transform indefinite amounts of substrate. There are, however, enzyme-like bodies known, the peroxidases of plants, in which there is a definite

mass relation between peroxidase and body oxidized, and it is not impossible that some luciferases behave in this way.

It should be pointed out in this connection that the light of these jellyfish comes unquestionably from granules of relatively large size. They can be seen at night under the microscope as dots of light with definite boundaries, not merely points of light. These granules shine brightly for some time, but if saponin or sodium glycocholate or fresh water is added, the granules dissolve with a sudden flare of light and then become dark. It is possible that the granule represents a combination of luciferin and luciferase in just the proper proportions for utilization. In *Cypridina* no such luminous granules exist in the extract (although granules occur in the luminous gland) or if they do exist in the extract they are ultra-microscopic in size.

It should also be borne in mind that Harden and Young[3] found an excess of zymase proper in some yeasts and an excess of co-zymase in other kinds. There is a certain resemblance between the luciferin-luciferase complex and the co-zymase-zymase complex and we may have an excess of luciferin in some animals and an excess of luciferase in others. Only if the latter condition existed could we demonstrate the presence of these two bodies.

I had hoped to solve this question by determining if the luciferin of medusæ will give light with the luciferase of some other form; *i.e.*, with a solution which we know to contain luciferase, as that of *Cypridina*. Such a test has given absolutely negative results. The luciferin of *Æquorea*, *Mitrocoma*, *Cavernularia* or *Ptylosarcus*, prepared in various ways, will give no light with *Cypridina* luciferase. Neither will the reverse "cross" (*Medusa* luciferase and *Cypridina* luciferin) give luminescence. These results are given in Table II.

This would seem to indicate that there was no *Mitrocoma* nor *Æquorea* nor pennatulid luciferin in the extract. There is a possibility, however, that *Cypridina* luciferase is absolutely specific and will not act with the luciferins of other forms. If that is the case, and my work shows that luciferin and luciferase are specific, except for very closely related forms, we cannot expect to throw light on the problem by this method.

[3] Harden, A., and Young, W. J., *Proc. Roy. Soc. Biol.*, 1906, lxxvii, 405; lxxviii, 369

There is a second method of attacking the problem. Suppose we prepare a solution which should contain medusa luciferin. On adding this to a glowing extract of medusæ, which must contain luciferin and also luciferase to oxidize the luciferin, a brighter light should result because within certain limits, with a given amount of luciferase, the more luciferin is present, the brighter will be the light. Trials have shown that no brighter light results from adding additional medusæ luciferin to a glowing medusa extract. Apparently, therefore, the medusa luciferin solution contains no luciferin or the glowing extract of medusæ contains no luciferase; in other words, these substances do

TABLE II.

"Cross."					Reaction.
Mitrocoma	luciferase	+ *Mitrocoma*	luciferin	.....	Negative.
"	"	+ *Cypridina*	"	.....	"
Æquorea	"	+ *Æquorea*	"	.....	"
"	"	+ *Cypridina*	"	.....	"
Cypridina	"	+ *Mitrocoma*	"	.....	"
"	"	+ *Æquorea*	"	.....	"
Cavernularia	"	+ *Cavernularia*	"	.....	"
"	"	+ *Cypridina*	"	.....	"
Cypridina	"	+ *Cavernularia*	"	.....	"
Ptylosarcus	"	+ *Ptylosarcus*	"	.....	"
"	"	+ *Cypridina*	"	.....	"
Cypridina	"	+ *Ptylosarcus*	"	.....	"
"	"	+ *Cypridina*	"	.....	Brilliant light.

not exist in the medusæ. While my work thus far points to this conclusion, we should certainly expect so fundamental a reaction as that of luciferin with luciferase to be universal. The statement that luciferin and luciferase do not occur in medusæ must therefore be considered as tentative and dependent on the present state of our knowledge.

Specificity of Luciferin and Luciferase.

The specificity of luciferin and luciferase is of considerable interest apart from the question discussed above. Accordingly, I have made a study of the luminescence resulting when *Cypridina* luciferin and luciferase is mixed with these bodies prepared from other animals of

TABLE III.

Organism.	"Cross."					Reaction.
Bacteria.	*Cypridina*	luciferase	+	*Bacteria*	luciferin.	Not tried.
	"	luciferin	+	*Bacteria*	luciferase.	" "
	Bacteria	luciferin	+	"	luciferase.	Negative.
Cystoflagellates	*Cypridina*	luciferase	+	*Noctiluca*	luciferin.	Negative.
	"	luciferin	+	"	luciferase.	"
	Noctiluca	luciferin	+	"	luciferase.	"
Medusæ.	*Cypridina*	luciferase	+	*Æquorea*	luciferin.	Negative.
	"	luciferin	+	"	luciferase.	"
	Æquorea	luciferin	+	"	luciferase.	"
	Cypridina	luciferase	+	*Mitrocoma*	luciferin.	"
	"	luciferin	+	"	luciferase.	"
	Mitrocoma	luciferin	+	"	luciferase.	"
Pennatulids.	*Cypridina*	luciferase	+	*Pennatula*	luciferin.	Negative.
	"	luciferin	+	"	luciferase.	"
	Pennatula	luciferin	+	"	luciferase.	"
	Cypridina	luciferase	+	*Cavernularia*	luciferin.	"
	"	luciferin	+	"	luciferase,	"
	Cavernularia	luciferin	+	"	luciferase.	"
	Cypridina	luciferase	+	*Ptylosarcus*	luciferin.	"
	"	luciferin	+	"	luciferase.	"
	Ptylosarcus	luciferin	+	"	luciferase.	"
Ctenophores.	*Cypridina*	luciferase	+	*Bolina*	luciferin.	Negative.
	"	luciferin	+	"	luciferase.	"
	Bolina	luciferin	+	"	luciferase.	"
Annelids.	*Cypridina*	luciferase	+	*Tomopteris*	luciferin.	Not tried.
	"	luciferin	+	"	luciferase.	Positive ?
	Tomopteris	luciferin	+	"	luciferase.	Not tried.
	Cypridina	luciferase	+	*Odontosyllis*	luciferin.	Negative.*
	"	luciferin	+	"	luciferase.	" *
	Odontosyllis	luciferin	+	"	luciferase.	Positive.
	Cypridina	luciferase	+	*Polynöe*	luciferin.	Negative.
	"	luciferin	+	"	luciferase.	"
	Polynöe	luciferin	+	"	luciferase.	"
	Cypridina	luciferase	+	*Chætopterus*	luciferin.	"
	"	luciferin	+	"	luciferase.	"
	Chætopterus	luciferin	+	"	luciferase.	"
Crustacea.	*Cypridina*	luciferase	+	*Pyrocypris*	luciferin.	Positive.
	"	luciferin	+	"	luciferase.	"
	Pyrocypris	luciferin	+	"	luciferase.	"

* Perhaps a slight reaction.

TABLE III—*Continued.*

Organism.	"Cross."					Reaction.
Crustacea.	*Cypridina*	luciferase	+	*Meganyctiphanes*	luciferin.	Negative.
	"	luciferin	+	"	luciferase.	"
	Meganyctiphanes	luciferin	+	"	luciferase.	"
Myriapods.	*Cypridina*	luciferase	+	*Geophilus*	luciferin.	Not tried.
	"	luciferin	+	"	luciferase.	" "
	Geophilus	luciferin	+	"	luciferase.	Negative (rather dilute solutions).
Insects.	*Cypridina*	luciferase	+	*Luciola*	luciferin.	Negative.
	"	luciferin	+	"	luciferase.	"
	Luciola	luciferin	+	"	luciferase.	Positive.
Molluscs.	*Cypridina*	luciferase	+	*Pholas*	luciferin.	Negative.
	"	luciferin	+	"	luciferase.	" (?).
	Pholas	luciferin	+	"	luciferase.	Positive.
Ascidians.	*Cypridina*	luciferase	+	*Pyrosoma*	luciferin.	Negative.
	"	luciferin	+	"	luciferase.	"
	Pyrosoma	luciferin	+	"	luciferase.	"
Fish.	*Cypridina*	luciferase	+	*Photoblepharon*	luciferin.	Negative.
	"	luciferin	+	"	luciferase.	"
	Photoblepharon	luciferin	+	"	luciferase.	"
	Cypridina	luciferase	+	*Anomalops*	luciferin.	"
	"	luciferin	+	"	luciferase.	"
	Anomalops	luciferin	+	"	luciferase.	"

all degrees of relationship as regards *Cypridina*. The results are best expressed in the form of a tabulation. In this table the words "luciferin" and "luciferase" are used to indicate extracts so prepared that they should contain luciferin and luciferase, provided these substances are formed by the organism in question. Table III represents the results of experiments performed at various times during the past seven years in all parts of the world. Its incompleteness is due to the difficulty of obtaining luminous animals in sufficient quantity for the test in question.

From the evidence in Table III it would seem that *Cypridina* luciferase will only give light with the luciferin of a very closely related form, *Pyrocypris*, a genus that differs from *Cypridina* only in the character of appendages on the upper lip. Both genera belong to the family, *Cypridinidæ*. Crosses of *Cypridina* luciferase with the luciferin of *Odontosyllis*, *Pholas*, or fireflies, all of which possess a luciferin-luciferase reaction, gives only negative or very faint positive results as does the reverse mixture, *Cypridina* luciferin crossed with the luciferase of these other forms. All my attempts to oxidize *Cypridina* luciferin with oxidizing enzymes or oxidizing agents of various kinds have failed, and also all attempts to oxidize with light production, various easily oxidizable substances with *Cypridina* luciferase.[4] We must therefore conclude that these two substances responsible for the production of light by *Cypridina*, are specific to the highest degree.

SUMMARY.

Among sixteen groups of luminous forms investigated by the author, in only four (fireflies, *Pholas*, ostracods, and *Odontosyllis*) is it possible to demonstrate the luciferin-luciferase reaction. In many groups this is probably due to the small amount of these substances present in the luminescent organism or to their instability. In the medusæ and pennatulids, despite a large amount of luminescent material, luciferin and luciferase cannot be demonstrated. This does not appear to be due to the presence of luciferin and luciferase in equivalent proportion, or to their instability. In fact, one is led to the conclusion that luciferin and luciferase do not exist in these forms, but such a conclusion must be regarded as merely tentative, in view of the fundamental character of the luciferin-luciferase reaction. Luciferin of one form will not luminesce with the luciferase of another form or *vice versa*, unless very closely related (*Cypridina* and *Pyrocypris*). All experiments emphasize the specificity of the light producing substances of *Cypridina*.

[4] Harvey, E. N., *J. Gen. Physiol.*, 1918–19, i, 269.

THE EFFECTS OF RADIUM RAYS ON METABOLISM AND GROWTH IN SEEDS.

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The view is current in the literature on the physiological action of radium radiations that these rays produce their effects by altering the metabolism of the tissues exposed to them. The basis for this view appears to be the observation that exposure to radium changes the rate of cell division and growth. If the term metabolism is to be used in describing the reaction of organized matter to any reagent, in order to attain analytical significance it must be defined in terms of some definite set of chemical transformations. We have consequently carried out a series of experiments designed to test the effect of the β-rays of radium on those metabolic processes which result in the production of CO_2 and to demonstrate whether any correlation exists between alterations produced in these activities and the ability of the protoplasm to take part in cell division and growth.

It is essential for such an experiment to select material which will enter into some well defined growth process, and of which the CO_2 production can be examined before any change in the bulk of protoplasm has brought about a significant alteration in the quantity of CO_2 produced. For this purpose we have made use of radish seeds, and have measured the CO_2 given off, before germination has had time to take place. Doubtless, cell division and growth were beginning to take place while the measurements were being made, but any changes in CO_2 production which occurred as a result were not sufficient to mask the effects produced by the radiations.

The output of CO_2 was measured in the apparatus described by Osterhout.[1] Fifty or seventy seeds were moistened and spread over the surface of a test-tube. Air was circulated between this test-tube

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17.

and another containing dilute NaOH and phenol red, and a measurement was made of the time required for the liberated CO_2 to change the reaction of this solution from pH 7.60 to pH 7.09. The rate of CO_2 production per seed was expressed by the reciprocal of this time divided by the number of seeds.[2] During the measurement the tube containing the seeds was immersed in a water bath at constant temperature, usually at 25°C.

In order to determine the accuracy of the method, and the variations which would result from measuring different groups of seeds a series of control experiments was performed. In each the seeds were divided into two numerically equal lots, and six or eight successive measurements made of the CO_2 production. Table I shows that the

TABLE I.

Comparison of Unradiated Seeds.

No. of seeds per lot.	CO_2 production per seed.*		Difference.
	Lot 1.	Lot 2	
			per cent
50	630± 6.8	610±11.0	± 3.1
50	696± 4.3	782±10.7	±11.0
50	1516±18	1470±20	± 3.2
50	286± 2.5	252± 2.6	±13.4
70	480± 2.67	354± 2.83	±35.6
70	458± 5.9	422±13.8	± 8.4

* Method of calculating explained in the text.

probable error of the mean of the successive measurements on any lot does not exceed as a rule 5 per cent. The percentage difference in measurements of different lots is less than 13 per cent in all but one of the experiments.

The ability of the seeds to germinate and grow was examined by placing comparable lots of seeds on moist filter paper. They were covered by a large glass vessel to prevent rapid drying, but access of fresh air was not completely cut off. It was found that at least 95 per cent of unradiated seeds germinated and grew under these conditions.

[2] The seeds were weighed in the dry condition and the CO_2 production estimated per gram of seeds. The relative CO_2 productions of different lots of seeds expressed in this way did not differ materially from those obtained by the method employed above.

The seeds were exposed to the β- and γ-rays of radium by placing them in the dry condition in a test-tube, closely packed about a glass tube containing radium emanation.[3] The α-rays failed to penetrate the walls of the tube; in comparison to the β-rays, the γ-rays doubtless contributed to the effect in negligible degree owing to their limited absorption in the seed. 2 days after radiation the seeds were moistened and the CO_2 production was determined. They were then given an opportunity to germinate and grow. In each experiment an equal number of unradiated seeds was kept as a control. Their CO_2 production was measured and they were then set to germinate on the same moist filter paper and under the same cover with the radiated seeds.

TABLE II.

Comparison between Radiated and Unradiated Seeds.

No. of seeds per lot.	CO_2 production per seed.*		Difference.	Amount germinated on 3rd day.	
	Radiated.	Control.		Radiated.	Control.
			per cent	*per cent*	*per cent*
54	851	748	+ 13.8	26	59
56	877	777	+ 12.9	19	81
50	753	544	+ 38.4	28	100
50	696	445	+ 56.2	16	72
70	445	370	+ 20.4	40	90
70	360	216	+ 66.5	23	98

* Method of calculating explained in text.

Table II, containing the results of six experiments, shows that the difference in the rate of CO_2 production between radiated and unradiated seeds is greater in almost every case than that found between comparable lots of unradiated seeds, and that *the rate of CO_2 production in radiated seeds is invariably greater than in the corresponding unradiated control.* In comparison to this it is seen that the germination of the radiated seeds is retarded to a marked degree. A notable feature is that the radiated seeds germinate much more slowly than normal for it usually requires 2 days before any of these seeds send out roots, and the number of seeds which have done so increases from day

[3] We are indebted to Dr. William Duane for placing a supply of radium belonging to the Cancer Commission of Harvard University at our disposal.

to day much more slowly than in the case of the controls. In addition, many seeds seem to be permanently prevented from germinating, so that at the end of a week the number which have commenced to grow is far below that found in a lot of unradiated seeds. The seeds which fail to germinate as the result of radiation are not killed, as is shown by the undiminished CO_2 production. Twenty-five radiated seeds from the radiated lot described in the fourth experiment of Table II which had failed to germinate after 7 days still produced CO_2 at a rate of 606 units per seed.

These results indicate that no direct relation exists between the effect of β-rays on CO_2 metabolism and growth in radish seeds. In this respect they recall the observation of Warburg[4] and Loeb and Wasteneys,[5] that by decreasing the hydrogen ion concentration of sea water, the O_2 absorption of echinoderm eggs increases and at the same time segmentation is prevented.

Kimura[6] has shown that if living carcinoma and sarcoma cells from the mouse are exposed *in vitro* to weak doses of x-rays the processes of oxidation which lead to CO_2 production are stimulated and that in the case of the sarcoma the growing power is increased to some extent. Stronger radiation diminishes the CO_2 production of both types of cell and destroys their power of mitotic division and growth. The important point which is demonstrated by the present experiments, in contrast to those of Kimura, is that the *changes in the rates of CO_2 production and cell division do not always go hand in hand; one may be increased by exposures which retard the other.* This conclusion is not as unexpected as it may appear at first, for the pathological changes which are produced in living matter by radiations, *i.e.* the malformation of embryos, must be due to an unequal effect on various processes which go on side by side. If all processes were affected alike their courses might be run more rapidly or more slowly, but at any instant the conditions of equilibrium in the tissue would not be abnormal. It is the specific action of radiations on certain physiological processes in contrast to others which accounts for the characteristics of their effects.

[4] Warburg, O., *Z. Physiol. Chem.*, 1910, lxvi, 305.
[5] Loeb, J., and Wasteneys, H., *J. Biol. Chem.*, 1913, xiv, 355.
[6] Kimura, N., *J. Cancer Res.*, 1919, iv, 95.

It is because radiations have a specific action on certain physiological processes in contrast to others that their characteristic effects are produced. Consequently it is unjustifiable to make any general assumption concerning their action on the chemical changes of living matter as a whole.

CORRESPONDENCE OF SKIN PIGMENTS IN RELATED SPECIES OF NUDIBRANCHS.

By W. J. CROZIER.

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(Received for publication, October 21, 1921.)

That pigmentary materials occurring in various species of certain genera of marine invertebrates may be chemically very similar, or even identical, has several times been suggested.[1] In some cases the presence of such chemically similar coloring matters, playing an important rôle in determining the appearance of the animals, has a distinct part in the theory of animal coloration, since, if substantiated, identity of composition as regards pigments may be significant for the evolutional origin of types of pigmentation (*e.g.* in so called "warning" coloration[2]). The pigmentation of the large nudibranch *Chromodoris zebra* has been studied from the standpoint of its possible adaptive quality,[3] and since it happens that coloring matters superficially corresponding to those in *Chromodoris zebra* are found in a good many species of this widely dispersed genus,[4] it is desirable to examine the degree of chemical similarity of these substances. The blue pigment which plays a conspicuous part in the coloration of these creatures has properties favorable for such study. A yellow pigment, also of frequent occurrence in the genus, belongs to the carotinoid "lipochrome" series, but because of the very general occurrence of such substances as pigmentary components derived from plant food, it is less significant for the present purpose.

[1] Briot, A., *Compt. rend. Soc. Biol.*, 1906, lxxxviii, 1157. Crozier, W. J., *Zool. Jahrb., Abt. Zool.*, 1915, xxxv, 233; *J. Biol. Chem.*, 1918, xxxv, 455; *Am. Naturalist*, lii, 552.

[2] Crozier, W. J., *Anat. Rec.*, 1919–20, vii, 349.

[3] Crozier, W. J., *Anat. Rec.*, 1919–20, vii, 349; *Proc. Nat. Acad. Sci.*, 1916, ii, 672.

[4] Crozier, W. J., *J. Physiol.*, 1913–14, xlvii, 491.

Solutions of the blue material were compared in the case of the three species: *Chromodoris zebra* from Bermuda, *Chromodoris porteræ* from La Jolla, California, and *Chromodoris universitatis* from Laguna Beach, California. An extract from specimens of any of these species in aqueous formaldehyde, is bluish purple in color, turned pink by acids at about pH 5.6, and changed to blue (with some precipitation of a greenish flocculent material) by alkali.[5] In some other qualitative respects the substances extracted from the three species also agree, notably in yielding positive tests for the presence of manganese, and in being decolorized, reversibly reduced, by putrefactive bacteria; but the most important data are derived from the absorption spectra. In formaldehyde solution the *Chromodoris zebra* pigment gives a narrow, sharply bounded absorption band centering on 620 to 622$\mu\mu$. In alcoholic extract, neutral or faintly acid, the absorption band likewise centers at 622$\mu\mu$.

The extracts are stable for at least several years (especially if shielded from bright sunlight), hence it is unnecessary to employ freshly prepared solutions of the pigments. *Chromodoris porteræ* pigment, in the comparison spectroscope, gave an absorption which could not be distinguished from that of *Chromodoris zebra*, and, as determined by successive dilutions, the absorption maximum was also at about 620$\mu\mu$. This is likewise true of the pigment of *Chromodoris universitatis*.

It is believed, consequently, that the blue pigment which has a special importance for the coloration of the genus *Chromodoris* is a very similar body, chemically, in widely dispersed species of this genus;[6] and since the habits of the various species differ considerably, it must be reckoned as a distinct probability, therefore, that the pigment has primarily no relation to the habits of these animals, but is an expression, merely, of the fundamental chemistry of the Chromodorid stock.

[5] *cf.* Crozier, W. J., 1916, *J. Biol. Chem.*, xxiv, 255. In alcoholic solution the green material is soluble.

[6] *Chromodoris porteræ* were obtained from the Scripps Institution, and *Chromodoris porteræ* and *Chromodoris universitatis* from Professor W. A. Hilton of Pomona College; the author is grateful for this assistance.

THE PHYSIOLOGY OF THE RESPIRATION OF FISHES IN RELATION TO THE HYDROGEN ION CONCENTRATION OF THE MEDIUM.*

By EDWIN B. POWERS.

(From the University of Nebraska, Lincoln.)

(Received for publication, October 31, 1921.)

INTRODUCTION.

Within recent years much has been added to the knowledge concerning the mechanism of the respiratory function of the blood. Haldane and Priestley (1905) have shown that, at least in the higher animals, the respiratory movements are affected by the carbon dioxide tension of the arterial blood. It has been shown definitely (Hasselbalch, 1912 and citations) that the exciting agent is the hydrogen ion concentration of the blood bathing the respiratory center.

Krogh and Leitch (1919) undertook to study the respiratory function of the blood of fishes in view of the knowledge of the influence of temperature upon the dissociation curve of oxyhemoglobin as investigated by Barcroft and Hill (1909) and Barcroft (1914). These workers found that the blood of the fish was especially adapted to its needs.

Certain marine fishes are known to react to a gradient of acidity and alkalinity (Shelford and Powers, 1915). It has been found that certain species react positively to a definite range of hydrogen ion concentration of the sea water, others are less definite in their reaction, and still others seemingly do not respond to differences in alkalinity and acidity (Powers, 1921).

In view of these facts experiments were undertaken to determine the ability of marine fishes to extract oxygen from the sea water at different hydrogen ion concentrations. Interest in this question was

* Studies from The University of Nebraska, No. 131.

further stimulated by the theory held by Roule (1915) that the salmon (*Salmo salar* L.) does not respond to salinity or temperature but that it always reacts in such a way as to bring it into water having a higher oxygen content.

Method.

A very simple technique was employed. A fish was placed in a 2 quart Mason jar filled with sea water and made air-tight with a rubber stopper. The jar was then immersed in a water bath of running sea water in which the temperature was almost constant. The oxygen content of the water at the beginning of an experiment was always sufficiently high so that the fish did not at first suffer from oxygen want. The hydrogen ion concentration was determined by the colorimetric method; and the oxygen, by the Winkler method immediately after all movements of the fish had ceased.

The hydrogen ion concentration of the sea water was varied by aerating with carbon dioxide-free air or by the addition of a small amount of sea water made alkaline by the addition of sodium hydroxide and by the introduction of carbon dioxide.

The fishes used in these experiments were kept in a small aquarium of running sea water. They were allowed to rest from 6 to 12 hours, so that they might recover from any shock suffered when collected. All were rejected after being in the laboratory 2 or 3 days so as to avoid as far as possible erratic results due to the ill effect of keeping them under unnatural conditions.

EXPERIMENTAL.

The mean oxygen content of the sea water of all experiments having approximately the same pH at the time of death of the fish was calculated. That is, if the highest oxygen content of the sea water at the time of death of the cunner at 7.22 to 7.25 pH was 0.30 cc. per liter, and the lowest 0.20 cc. per liter, the cunner was taken to be able to absorb the oxygen from the sea water down to 0.25 cc. per liter at a pH from 7.22 to 7.25. This was taken to be more nearly exact and to eliminate to a greater extent the factor of individual variation than if individuals were taken alone. Loeb (1912) has shown that the mortality curve of *Fundulus* embryos under the in-

fluence of acids, alkalies, and potassium salts, and Loeb and Northrop (1917) that the mortality curve of the fruit fly (*Drosophila*) under the influence of food and temperature are probability curves. The probable cause of the variations in the ability of different individual fish of the same species to absorb oxygen from the medium at low oxygen pressure at a given hydrogen ion concentration and temperature will be taken up later.

The figures are graphic representations of the experimental data.

The Ability of Fishes to Extract Oxygen from the Sea Water at Different Hydrogen Ion Concentrations.

(*a*) *The Cunner* (*Tautogolabrus adspersus* Walbaum).—The cunner was able to extract oxygen from the sea water down to approximately 0.25 cc. per liter before asphyxiation at all hydrogen ion concentrations up to about 6.5 pH. From this point on there seems to be a rapid loss in the ability of this fish to extract oxygen from the sea water at low oxygen pressure (Fig. 1).

(*b*) *The Butter-Fish* (*Poronotus triacanthus* Peck).—Fig. 2 shows that the butter-fish was not able to extract the oxygen from the sea water at quite as low a level as the cunner. The general trend of the curve indicates that the butter-fish is able to absorb oxygen at the lowest concentrations at a pH of about 7.6 to 7.8. This was shown even more strikingly when individuals were considered, the lowest point at which oxygen was absorbed being 0.36 cc. per liter at 7.58 pH and 0.35 cc. per liter at 7.57 pH. There was a slight falling off in the power to extract the oxygen at low concentrations, as the hydrogen ion concentration of the sea water was lowered from a pH of about 7.6 or 7.8 to 9.0. The falling off was much more rapid when the hydrogen ion concentration was increased from a pH of about 7.6 to 6.3, being most rapid from 7.1 to 6.35. At the higher hydrogen ion concentrations but very little of the oxygen had been absorbed before asphyxiation took place.

(*c*) *The Mackerel* (*Scomber scombrus* L.).—There was only a slight variation in the ability of the mackerel to extract oxygen from the sea water at low tension from about 7.7 pH to about 8.2 pH within an optimum at about 8.0 pH. There was a more or less falling off in

this ability when the pH of the water was either raised above or lowered below this range (Fig. 1).

(*d*) *The Alewife* (*Pomolobus pseudoharengus* Wilson).—The optimum pH for the absorption of oxygen at low oxygen tension for the

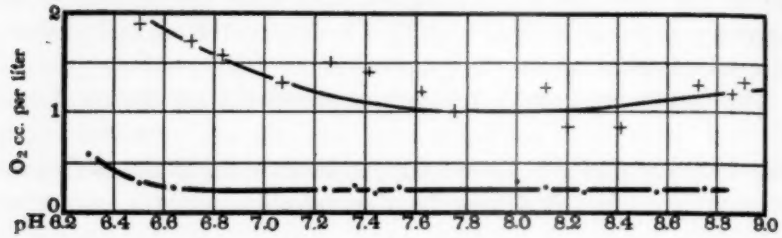

FIG. 1. The circles represent the experimental data of the cunner (*Tautogolabrus adspersus* Walbaum) and the plus signs those of the mackerel (*Scomber scombrus* L.). The abscissæ give the hydrogen ion concentration; and the ordinates the oxygen in cc. per liter at the end of the experiment.

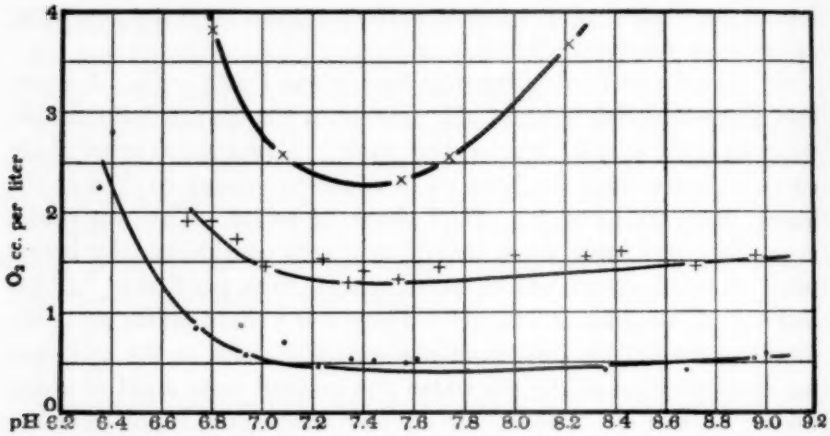

FIG. 2. The circles represent the experimental data of the butter-fish (*Poronotus triacanthus* Peck), the plus signs those of the alewife (*Pomolobus pseudoharengus* Wilson) and the crosses those of the herring (*Clupea harengus* L.). The abscissæ give the hydrogen ion concentration; and the ordinates the oxygen in cc. per liter at the end of the experiment.

alewife was about 7.3 or perhaps 7.3 to 7.6 (Fig. 2). The falling off from this optimum was greatest when the hydrogen ion concentration was raised above this optimum. The falling off was less marked when the hydrogen ion concentration was lowered.

(*e*) *The Herring* (*Clupea harengus* L.).—The herring (?) used in these experiments averaged from 3 to 4 gm. This fish appeared in great numbers for only a few days. Their sudden disappearance terminated the experiments and prevented the writer from having his identification of the species verified by a specialist. The young of the alewife were running at the same time and were taken by members of the U. S. Bureau of Fisheries.

The herring (Fig. 2) showed greater differences in ability to absorb oxygen at low oxygen tension with variation in the hydrogen ion concentration of the sea water than any of the other fishes tested. There was a very rapid falling off of the power to absorb oxygen from the sea water when the hydrogen ion concentration was raised at least above a pH of 7.4. It was less rapid when the hydrogen ion concentration was lowered.

The experiments just described show that the lowest tension at which oxygen can be absorbed from the sea water by certain marine fishes is more or less dependent upon the hydrogen ion concentration of the sea water. Of the five species of fishes tested, the cunner, butter-fish, mackerel, alewife, and herring, the cunner and butter-fish were affected least by a change in the hydrogen ion concentration. The lowest point at which the butter-fish was able to absorb oxygen from the sea water was at 0.35 cc. per liter at a pH of 7.57.[1] The mackerel, alewife, and herring were most affected by the variation in the pH of the sea water; *i.e.*, have the narrowest optimum pH. These fishes are considered the most sensitive and most restricted in their migratory movements. On the other hand, the cunner which is least affected is more cosmopolitan as to habitat and is an all year resident (Sumner, Osburn, and Cole, 1911). The butter-fish which shows less resistance to the variation in the pH is somewhat less cosmopolitan than the cunner in its habitat and is a resident of the Woods Hole vicinity only from early summer to late fall.

[1] This could not be called the optimum pH of the sea water for carrying on the respiratory functions by this fish. At the same time oxygen is being absorbed from the sea water bathing the gills, carbon dioxide is being given off. The pH at the beginning of the experiment was 8.28. Thus it stands to reason that the optimum pH of the sea water for this fish would be some where between 8.28 and 7.57.

When the figures are examined more closely it is seen that the optimum pH for the absorption of oxygen at low tension varies with the different species. The herring has the lowest pH optimum which seems to be correlated with the habits of this fish.

Results of Experiments on the Pacific Herring (*Clupea pallasii* Cuv. and Val.).

The foregoing experiments were performed at the Marine Biological Laboratory, Woods Hole, during the summer of 1920. It was thought desirable to test the Pacific herring, *Clupea pallasii*, in the same manner since its behavior was better known (Shelford and Powers, 1915 and Powers, 1921) than the Atlantic herring *Clupea harengus*. These experiments were performed at the Puget Sound Biological Station, Friday Harbor, during the summer of 1921. The methods employed in the experiments were similar to those at Woods Hole. In order to eliminate individual variation and make the time until death shorter, five 1.5 to 2.5 gm. fish were put in a 2 quart Mason jar of sea water instead of only one. The oxygen was determined immediately after the last fish had died. A total of three hundred and seventy-five fish were tested. Instead of taking the means of the extremes of the oxygen content at the end of an experiment, as was done in the preceding experiments, a curve, which is the mathematical mean of all experiments, was drawn (Fig. 3). The greatest deviation from the mean was 0.18 cc. per liter with the exception of three experiments. In two, one at 6.95 pH where the fish died when the oxygen content was 1.08 cc. per liter and another where the pH was 7.88 and the oxygen content was 0.98 cc. per liter at the end of the experiment, the variation from the mean was plus 0.25 cc. per liter. These two experiments seemed somewhat erratic but they have been included in all averages and calculations. In another, where the pH was 7.43, the variation from the mean was 0.20 cc. per liter. The average variations were plus 0.089 and minus 0.085 cc. per liter. This is less than 2 per cent of the total oxygen content of the sea water at the beginning of the experiments and less than 12 per cent of the difference of the extremes of the oxygen content of the sea water at the time of death of the fish. The

extreme variation from the mean is less than 33 per cent of the total variation in the experiments.

Duplicate oxygen tests could not be made by the methods employed since only 2 quarts of sea water were used in each experiment. However, the accuracy of the method was determined in connection with other work done at the same time. In 88 duplicate oxygen tests, the average deviation from the mean was ± 0.024 cc. per liter. This is more than 27 per cent of the average variation of the oxygen content at end of experiment from the mean curve (Fig. 3). The extreme

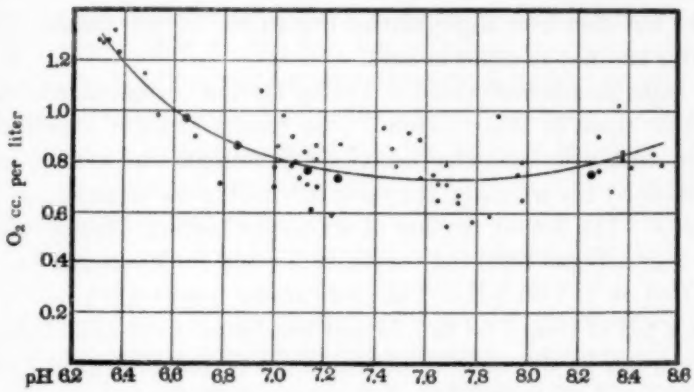

FIG. 3. The small circles represent the experimental data of the herring (*Clupea pallasii* Cuv. and Val.) when five fish were tested in a 2 quart mason jar, and the large circles when ten to twenty-five fish were tested in a 42 liter carboy. The abscissæ give the hydrogen ion concentration; and the ordinates the oxygen in cc. per liter at the end of the experiment.

deviations of the 88 duplicate tests were one 0.094 cc. per liter and four from 0.078 to 0.072 cc. per liter. This, again, is more than 33 per cent of the extreme deviations of the experiments from the mean curve. In other words, from 27 per cent to 33 per cent of the deviation of the experiments are probably due to the method employed and not the individual variation of the fish. Thus the average individual variation of the fish is less than 8 per cent and the extreme is less than 22 per cent of the difference of the total variations of the experiments. Further justifications for the form of the curve will be taken up in connection with the discussion of other experiments.

In order to determine whether or not the death of the fish was due to asphyxiation because of low oxygen tension of the sea water, the first set of experiments was checked by testing the fish where they would not suffer from oxygen want so quickly. Ten to twenty-five fish were placed in a carboy (capacity about 42 liters) of sea water at different hydrogen ion concentrations. The results of these experiments conform very markedly to the mean curve, although the time until death of the fish in the carboy was from 24 to 48 hours as compared with 1 hour and 23 minutes to 2 hours and 40 minutes when five fish were tested in the 2 quart Mason jars (Fig. 3). This shows that the fish died from asphyxiation due to low oxygen tension in the sea water and not to other causes.

The experiments performed at Friday Harbor corroborate in every way those done at Woods Hole. The results of these experiments conform strikingly with the behavior experiments and field observations made by the writer on the same fish during the summers of 1918 and 1919. The lowest tension at which the herring, *Clupea pallasii*, can extract oxygen from the sea water is when the hydrogen ion concentration is ±7.68 pH. This fish reacts positive to sea water having a pH of from 7.68 to 7.73 and was found most abundantly in the sea water in the vicinity of the Puget Sound Biological Station that had a pH of about 7.73 to 7.76 (Powers, 1921).

DISCUSSION.

Workers on fish respiration have found that fishes are able to survive at rather low oxygen tension without any apparent ill effect (Powers, 1921, citations). Gaarder (1918) found that the actual oxygen consumption of an anesthetized carp was reduced only slightly, 0.62 cc. per kg. per minute, until the oxygen in solution in the water had been reduced from 15.4 cc. per liter to about 1.13 cc. per liter when the gills were artificially bathed with water at a constant rate. It is not known whether or not this slight falling off of oxygen consumption could have been corrected had the respiratory mechanism of the fish been free to respond normally. Packard (1905) showed that *Fundulus heteroclitus* injected with 5/16 M sodium carbonate were able to live longer in a liter flask of sea water

stopped tightly to exclude air than those not so injected. He also found that when the fish were injected with M/250 to M/500 solution of acetic acid, they did not live as long as the control fish. From these experiments Birge and Juday (1911) suggested that "if a fish possessed the power to alter the composition of its blood somewhat, it would be able to adapt itself to water which contains only a comparatively small amount of dissolved oxygen." Might it not be possible in these experiments, since the oxygen tension was lowered by the respiration of the fish, that the differences in survival time of the fish were due to the variations in their abilities to absorb oxygen from the sea water at low oxygen tension under the conditions of the experiment? That is, the fish whose blood was best adapted to absorb oxygen at low tension at the particular carbon dioxide tension of the experiment would survive longest. This ability would perhaps depend upon the alkaline reserve of the blood of the fish. That is, if the hemoglobin of the blood of all the fish of a given species had the same optimum pH to carry oxygen, the fish would be able to extract oxygen from the water at low oxygen tension in the direct order of the alkaline reserve of the blood at a carbon dioxide tension higher than the optimum and in the reverse order of the alkaline reserve in a carbon dioxide tension below the optimum. In these experiments the carbon dioxide tension would tend to increase above the optimum for the absorption of oxygen at low tension since ordinary sea water was used and, as the oxygen was absorbed by the confined fish, carbon dioxide would be given off.[2]

The experiments recorded in the figures show that the species of fish tested have an optimum pH of more or less narrow or wide range for absorbing oxygen from the sea water at low oxygen tension. The limiting factor is perhaps the carbon dioxide tension of the sea water. The pH of the sea water with a given alkaline reserve is dependent upon the carbon dioxide tension (Henderson and Cohn, 1916, and McClendon, 1917). The optimum carbon dioxide tension of the sea water for a species of fish as suggested above would perhaps depend upon the alkaline reserve and the optimum pH of its blood.

[2] The author has performed experiments which give strong evidence for this probability, which are reserved for further publication.

The individual variation of fish of a given species might depend upon the individual variation in the alkaline reserve of the blood.

These conclusions are further emphasized by FitzGerald's observations (1913, 1915), and recent experiments by Henderson (1919), Haggard and Henderson (1920), and Henderson (1920) on the part played by the alkaline reserve of the blood in the acclimatization to altitude. It might be further suggested that the species of fish having the greatest ability to vary the alkaline reserve of their blood would also have the greatest power to withstand a variation in carbon dioxide tension of the water. The rapidity of the change in the carbon dioxide tension which a fish would be able to withstand would depend upon the rapidity with which it is able to vary the alkaline reserve of its blood. In man (Henderson, 1920) this seems to be a rather slow process. This is a more logical explanation since the carbon dioxide and oxygen tension in the environment of the fish are not so intimately connected as with the air-breathing animals. But as a general rule when the oxygen content of the sea water is low the carbon dioxide is increased (Powers, 1920). However, further experiments are necessary to settle these points.

In the light of these experiments it is easily seen why Wells (1913) found that high carbon dioxide was more rapidly fatal to fishes than low oxygen and why fishes are able to sense out and detect variations in carbon dioxide tension more easily than the variations in the oxygen tension (Shelford and Allee, 1913). Bayliss (1918) states: " . . . . . if increase in carbon dioxide be prevented, as by respiration of pure nitrogen, a man may become unconscious before experiencing any unpleasant symptoms."

It is conceivable that the carbon dioxide tension of the water could be raised above the carbon dioxide tension of the tissue capillary blood of the fish. Under this condition the oxyhemoglobin would be reduced to hemoglobin in the capillaries of the gills of the fish rather than the hemoglobin being oxidised to oxyhemoglobin. The fish would then suffer oxygen want more quickly in water with a high carbon dioxide tension and ordinary oxygen tension than in water with a low carbon dioxide tension and a low oxygen tension. Thus the probabilities are that in Wells' experiments the high oxygen tension was not antagonistic to the high carbon dioxide tension, in

the true sense of the word, but that the oxygen tension was below on the one hand, and on the other it was above the tension at which hemoglobin could be oxidised to oxyhemoglobin in the presence of a high carbon dioxide tension.

SUMMARY.

1. The ability of marine fishes to absorb oxygen at low tension from the sea water is more or less dependent upon the hydrogen ion concentration of the water.

2. The ability of fishes to withstand wide variations in the range of hydrogen ion concentration of the sea water can be correlated with their habitats. The fishes that are most resistant to a wide variation in the hydrogen ion concentration are most cosmopolitan in their habitat. Those that are least resistant to a variation in the hydrogen ion concentration are the most restricted in their range of habitat.

3. There is a close correlation between the optimum condition of the sea water for the absorption of oxygen at low tension by the herring (*Clupea pallasii*), the condition of the sea water to which they react positive and that in which they are found most abundantly.

4. It is suggested that the variation in the ability to absorb oxygen at low tension at a given pH of individuals of a species is dependent upon the alkaline reserve of the blood of the individual fish.

The author wishes to thank Professor Frank R. Lillie, Director, and Mr. G. M. Gray, Curator, of the Marine Biological Laboratory, and Professor T. C. Frye, Director of the Puget Sound Biological Station, for rooms, equipments, and materials for this work, and for many courtesies during its progress. The author wishes further to thank Professor Jacques Loeb for suggestions in the presentation of data, and Dean R. A. Lyman for reading manuscript.

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THE EFFECT OF IODINE AND IODOTHYRIN ON THE LARVÆ OF SALAMANDERS.

IV. The Rôle of Iodine in the Inhibition of the Metamorphosis of Thymus-Fed Salamanders.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

Received for publication, November 2, 1921.)

In previous papers I have shown that feeding thymus gland to the larvæ of salamanders retards or completely inhibits metamorphosis[1,2] as well as growth.[3] It was also demonstrated[2] that the inhibition of both growth and metamorphosis is not caused by specific growth-inhibiting substances contained in the thymus, but by a deficiency of the thymus in certain substances which are necessary for growth and metamorphosis. Later I suggested that these substances might be identical with iodine.[4]

Recently, however, I found[5] that inorganic iodine even when administered in excessive quantities does not accelerate either growth or metamorphosis of salamander larvæ. Although this result does not necessarily mean that salamander larvæ can grow and metamorphose in the complete absence of iodine, the correctness of my assumption that inhibition of growth and metamorphosis of thymus-fed salamander larvæ may be caused by a deficiency of the thymus in iodine, became doubtful, and special experiments were carried out to test this assumption. In the light of these experiments which will be briefly reported below, it appears that the substances which are deficient in the food of thymus-fed salamander larvæ are not identical with iodine.

[1] Uhlenhuth, E., *Proc. Soc. Exp. Biol. and Med.*, 1917-18, xv, 37.
[2] Uhlenhuth, E., *J. Gen. Physiol.*, 1919, i, 305.
[3] Uhlenhuth, E., *J. Exp. Zool.*, 1918, xxv, 141.
[4] Uhlenhuth, E., *J. Gen. Physiol.*, 1919, i, 473.
[5] Uhlenhuth, E., *Endocrinology*, 1922 (In press); *Biol. Bull.*, 1922 (In press).

Of three series of larvæ of *Ambystoma maculatum* from the same brood, Series 1 was fed earthworms and kept in iodine-free water, Series 2 was fed thymus and kept in iodine-free water, Series 3 was fed thymus and kept in water to which, from the 26th day on, 1 drop of a 1/20 M solution of inorganic iodine per 1000 cc. of water had been added.

Growth began to decrease in both thymus-fed series at an age of 19 days and practically ceased at an age of 26 days. From this time on inorganic iodine was administered to Series 3 as stated above, but did not improve the growth of this series at all. At the 54th day both thymus-fed series were divided into two lots and, in order to make sure that the ineffectiveness of the iodine was not due to a loss of the ability to grow, earthworms were fed instead of thymus. The result was as follows: In Series 2 the larger and stronger larvæ were continued on thymus; they did not grow, and finally died at an age of 82 days. The smaller larvæ received earthworms instead of thymus; they immediately began to grow, reached a normal size and finally metamorphosed. In Series 3 to which the iodine was administered the smaller larvæ received earthworms instead of thymus; this change of the diet again resulted in vigorous growth and in metamorphosis. The larger larvæ were continued on thymus, but in spite of the administration of iodine did not grow at all, until, at the 82nd, day, earthworms were used as food instead of thymus. This diet again resulted in normal growth and metamorphosis.

The objection could be raised that the amount of iodine was too small to permit of growth and metamorphosis. It is possible that the concentration of iodine was not high enough to permit of normal growth and metamorphosis. If, however, a lack of iodine had anything to do with the inhibition of growth and metamorphosis, even the slightest amount of iodine should have produced an improvement. Since even no improvement could be obtained by iodine-administration, but immediately followed the administration of earthworms, the conclusion seems to be justified that the inhibition of growth and metamorphosis of thymus-fed salamander larvæ is not caused by a deficiency of the thymus in iodine, but by a deficiency in certain substances, as yet unknown, which are contained in the earthworm.

THE INFLUENCE OF FEEDING THE ANTERIOR LOBE OF THE HYPOPHYSIS ON THE SIZE OF AMBYSTOMA TIGRINUM.

By EDUARD UHLENHUTH.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 19, 1921.)

In a previous article[1] I have shown that feeding the anterior lobe of the hypophysis of cattle to metamorphosed salamanders not only produces a far higher rate of growth than does the feeding of earthworms, but causes the animals to grow beyond the known maximum size of the species. The purpose of the present paper is to report on the results of a continuation of the experiments on *Ambystoma tigrinum*.

Hypophysis-Fed Animals Compared with Normal (Worm-Fed) Animals.

Figs. 1 and 2 show the animals of Experiment 1 at an age of 88 weeks. In Fig. 1 are shown the three worm-fed controls, in Fig. 2 the two hypophysis-fed animals. Total length, weight, and sex are indicated in the figure legends. In comparing the controls with the experimental animals it will be noticed that hypophysis-feeding resulted in a larger size than the feeding of normal food. Figs. 3 and 4 show that a similar relation between the controls and the hypophysis-fed animals has persisted up to the day that the animals reached an age of 132 weeks. Fig. 3 shows the worm-fed controls of Experiment 1 at an age of 132 weeks, Fig. 4 the hypophysis-fed male (the female died) at the same age. Comparison of the photographs as well as of the figures for size and weight demonstrates that the hypophysis-fed animal is still by far the largest one.

A second experiment gave the same result. Fig. 5 shows the controls of Experiment 2; Fig. 6, the hypophysis-fed animals of the same experiment at an age of 88 weeks. Again the hypophysis-fed animals

[1] Uhlenhuth, E., *J. Gen. Physiol.*, 1920–21, iii, 347.

are much larger and heavier than the worm-fed controls. The hypophysis-fed animals were continued on a diet of anterior lobe and increased at the same rate as the hypophysis-fed animals of Experi-

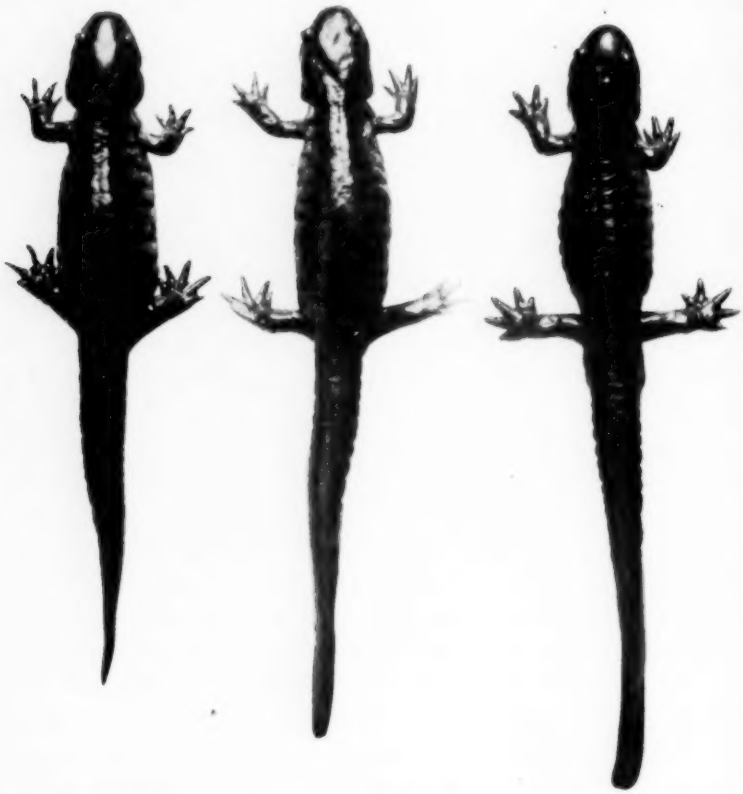

FIG. 1. Experiment 1. Worm-fed control animals at an age of 88 weeks No. 1, female (total length, 177.5 mm.; weight, 34.8 gm.); No. 2, female (total length, 194.5 mm.; weight, 40.0 gm); No. 3, male (total length, 200.5 mm.; weight, 35.0 gm.).

ment 1; they are shown in Fig. 7. All hypophysis-fed animals exceed the size of the largest known animal (235 mm.), the largest one being 25.1 per cent larger than the largest known animal. None of the worm-fed control animals has reached as yet the size of the largest known animal.

FIG. 2. Experiment 1. Anterior lobe-fed animals, at an age of 88 weeks. No. 4, male (total length, 273.5 mm.; weight, 72.3 gm.); No. 5, female (total length, 235.5 mm.; weight, 78.0 gm.).

Hypophysis-Fed Animals Compared with Liver-Fed Animals.

Several food substances besides earthworms had been tried out, but none of them gave higher rates of growth or produced larger animals

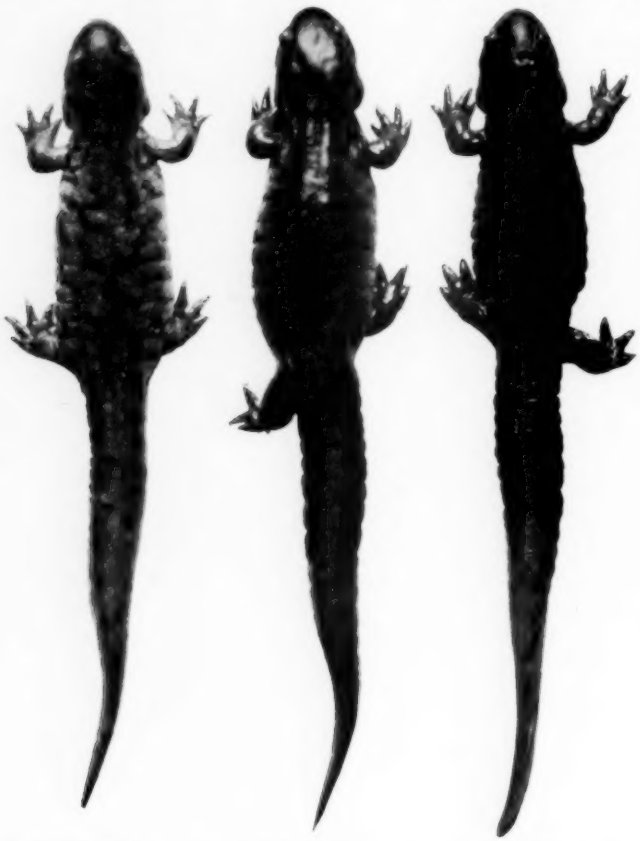

FIG. 3. Experiment 1. Worm-fed controls, at an age of 132 weeks. No. 1, female (total length, 200.8 mm.; weight, 57.0 gm.); No. 2, female (total length, 215.1 mm.; weight, 65.0 gm.); No. 3, male (total length, 212.0 mm.; weight, 55.0 gm.).

than did earthworms. Finally it was decided to use beef liver as food. The three worm-fed animals (Fig. 5) of Experiment 2 were used for this experiment. The two larger animals, male No. 1 and female No. 2, were fed liver, the smallest animal, male No. 3, was fed

FIG. 4. Experiment 1. Anterior lobe-fed animal, at an age of 132 weeks No. 4, male (total length, 294.0 mm.; weight, 108.0 gm.). No. 5 died; it measured 250.0 mm. when measured the last time (at an age of 126 weeks).

anterior lobe. The result is shown in Fig. 8. The hypophysis-fed male (to the right in Fig. 7), although it was by far the smallest animal before anterior lobe was fed (see Fig. 5), is now, after a period of ante-

FIG. 5. Experiment 2. Worm-fed controls, at an age of 88 weeks. No. 1, male (total length, 204.1 mm.; weight, 38.5 gm.); No. 2, female (total length, 186.6 mm.; weight, 40.2 gm.); No. 3, male (total length, 181.5 mm.; weight, 31.1 gm.).

rior lobe-feeding of only 37 weeks, nearly just as large as, and heavier than the liver-fed control, male No. 1 (the difference in weight was less pronounced a week before but has increased during a period of starvation to which the animals were subjected in preparation for an operation). Moreover the liver-fed animals, although they were

growing in the beginning at the same rate as the hypophysis-fed animals, have stopped growing during the last 26 weeks, while the

FIG. 6. Experiment 2. Anterior lobe-fed animals, at an age of 88 weeks; No. 4, male (total length, 257.1 mm.; weight, 95.0 gm.); No. 5, female (total length, 226.2 mm.; weight 84.3 gm.).

hypophysis-fed male, although it is of the same age and nearly of the same size as the liver-fed control, male No. 1, is still growing with

FIG. 7. Experiment 2. Anterior lobe-fed animals, at an age of 132 weeks; No. 4, male (total length, 285.0 mm.; weight, 108.0 gm.); No. 5, female (total length, 245.0 mm.; weight, 108.0 gm.).

FIG. 8. Experiment 2. Two of the controls fed liver and one fed anterior lobe for 37 weeks. No. 1, male (total length, 247.0 mm.; weight, 65.5 gm.); No. 2, female (total length, 212.0 mm.; weight, 61.0 gm.); No. 3, male (total length, 244.0 mm.; weight, 78.0 gm.; this animal was the smallest of the three before hypophysis-feeding started, as seen in Fig. 5).

considerable vigor. This condition may be taken as an indication that the hypophysis-fed animal will finally reach a size much in excess of the size of the liver-fed animals. The same result was obtained in two other experiments.

The liver diet not only permits a higher rate of growth than that resulting from an earthworm diet, but produces as high a rate of growth as that caused by anterior lobe-feeding. There is, however, an important difference between the effects of liver and hypophysis. The liver-fed animals stop growing a short while after liver-feeding begins, while the hypophysis-fed animals continue to grow at a high rate. That age and size have nothing to do with this difference, is clearly demonstrated by the fact that the hypophysis-fed male No. 3 of Experiment 2 is nearly as large (see Fig. 8) and just as old as the liver-fed male of the same experiment. The same result was obtained in two other experiments.

An inspection of the photographs shows that the liver-fed animals not only grow larger than the worm-fed animals, but exceed the known maximum size of normal animals of the species. This is the case with the liver-fed male, No. 1, of Experiment 2 (Fig. 8), which is 4.7 per cent larger than the largest known normal animal. It seems, however, that liver does not permit growth beyond a certain size far below that which hypophysis-fed animals attain.

SUMMARY.

1. Animals of the species *Ambystoma tigrinum* when fed anterior lobe can reach a size far in excess of that of animals fed earthworms and presumably also of that of liver-fed animals.

2. Liver produces a rate of growth as high as that resulting from anterior lobe-feeding, but maintains growth only, until the animals reach a definite size far below that of anterior lobe-fed animals.

THE TEMPERATURE COEFFICIENT OF PHAGOCYTOSIS.

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(Received for publication, November 21, 1921.)

Recently Madsen and Watabiki (1) have made some accurate measurements of the effect of temperature on the phagocytosis of bacteria. Fig. 1 is a reproduction of one of their figures showing the time curves of the number of bacteria ingested per leucocyte at different temperatures. In analyzing these results they endeavored to apply the familiar formula for a monomolecular reaction. In this formula, $K = \frac{1}{T} Log \frac{A}{A - x}$, they took A equal to the maximum number of bacteria ingested at the close of the experiment (instead of the maximum number of bacteria present, which, one is led to infer, was larger than A even at the higher temperatures) and x equal, as usual, to the number of bacteria ingested in time, T.

Considering their method of analysis it is not surprising that they found it impossible to calculate the temperature coefficient of phagocytosis from their figures. Inspection of Fig. 1 shows that the total number of bacteria ingested is smaller at the lower temperatures, *i.e.*, A is itself a function of temperature, increasing with rise of temperature. Now the accelerating effect of the higher temperatures is evidenced quite as much by the increase in A as by the increase in K, as they calculated it. The former factor they have completely discounted by their procedure, which is equivalent to "telescoping" the curves in Fig. 1 on the ordinate so that they all reach the maximum at the same point, and then comparing the times necessary for the different curves to reach the same ordinate. Moreover, the agreement of the experimental data with the formula for a monomolecular reaction was admittedly unsatisfactory at temperatures above 25°C. where the formula for a bimolecular reaction was usually found to give better results. But even if the results could be expressed

by the formula for a monomolecular reaction it could hardly be more than a meaningless coincidence. It could not indicate a rate proportional to the diminishing numbers of free bacteria because many bacteria were still present, at least at the lower temperatures, when phagocytosis had decreased to zero.[1] Nevertheless, the approximate empirical applicability of this formula to their results impressed the authors with the fact that phagocytosis obeys known physico-chemical

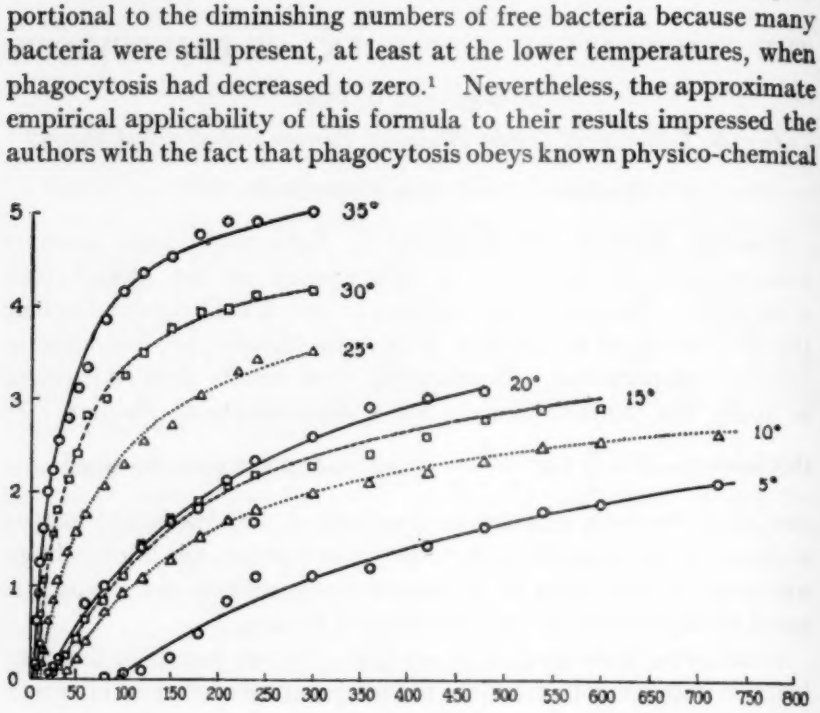


FIG. 1. Reproduction of curves from data of Madsen and Watabiki[2] showing the number of bacteria taken up per leucocyte (ordinates) as a function of time (abscissæ) at different temperatures. Note the shorter latent period, higher maximum and shorter duration of the experiment at higher temperatures.

laws. Actually, the attempt to force the experimental results to fit a known formula has merely beclouded the issue. It is worth while trying whether a more rational analysis of these curves will yield a more significant result.

Osterhout (3), in discussing the analysis of time curves, has pointed

[1] In a prevous paper on phagocytosis (2) the writer found that the rate of ingestion of solid particles by leucocytes was proportional to the decreasing number of available particles, thus giving a constant K when calculated by the formula for a monomolecular reaction.

out that one should compare the times necessary for equal amounts of action rather than the amount of action at equal times. This is equivalent to saying that the rates of two reactions should be compared at *corresponding stages*. In the curves of Fig. 1, however, the corresponding stages are not points of equal amount of action because the maximum varies at different temperatures, but, rather, they are points of equal percentages of the total amount of action possible at that temperature. For comparative rates, therefore, we may take the *number of bacteria ingested per leucocyte per minute during the first half of the reaction; i.e.*, until one-half the maximum number of bacteria has been ingested. This criterion yields a value for the rate of the reaction which is far from being ideal but which seems to be the best approximation possible under the circumstances and certainly more rational than the original.

Following this procedure the rates of the reactions at different temperatures have been calculated from the data of Madsen and Watabiki.[2] In order to calculate from them the temperature coefficient, Q_{10}, of the reaction the logarithms of these rates have been plotted in Figs. 2 and 3 against the corresponding temperatures. The temperature coefficient for any interval of 10 degrees on the abscissæ is the antilog of the difference between the ordinates at the two temperatures; *i.e.*, the slope of the graph for that interval. The resulting graphs are practically straight lines which is rather an unusual result for biological processes. This means that the temperature coefficient is constant over the entire range from 5°–35°C. Q_{10} was found to be 2.05 ± 5 per cent and 2.0 ± 5 per cent in Figs. 2 and 3 respectively. For comparison with these curves the values of K, calculated by Madsen and Watabiki according to the formula for a monomolecular reaction, are also plotted in Figs. 2 and 3. Our improved analysis evidently gives a smoother curve, the probable error being only about one-half as large.

In a recent paper on phagocytosis (2) a formula was derived for calculating the chances of collision between leucocytes and particles of known size and density when stirred together in a common suspen-

[2] Madsen and Watabiki (1), Tables 1 and 2. The data on other tables could not be similarly treated because the experiments were not carried to completion.

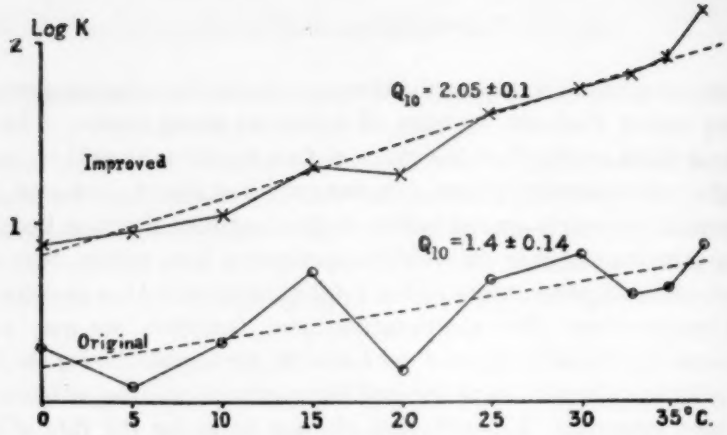


FIG. 2. Plots of the logarithms of K, the rate of phagocytosis (ordinates), against temperature (abscissæ). Data taken from Table 1 of Madsen and Watabiki. Lower curve from their own calculation of K by the formula of a monomolecular reaction. Upper curve shows the calculation of K by the improved method described in the text after correcting for viscosity of the medium. Slope of the graph (dotted line) represents the temperature coefficient of the process, Q_{10}, which is practically constant over this range of temperature. Calling the dotted line 100 per cent, the probable percentage deviation of the points from this line is only one-half as great by the new analysis. Before correcting for viscosity the new analysis gives higher values of Q_{10} than the original. Constants have been added to the values of Log K in both experiments for convenience in plotting.

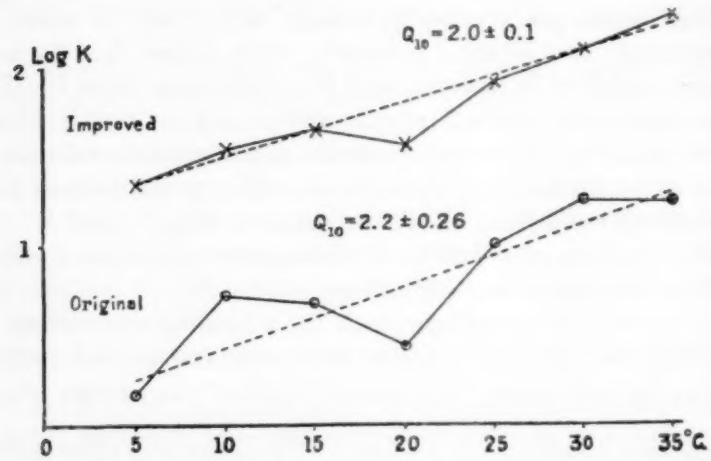


FIG. 3. Same as Fig. 1, but the data tak from another similar experiment Madsen and Watabiki[2].

sion. Since this formula was based on Stokes's law for falling bodies, the chances of collision are inversely proportional to the viscosity of the medium. In the previous experiments the viscosity could be neglected because it did not vary. Where temperature is the variable, however, it is obvious that the viscosity becomes a factor of some significance because it decreased proportionally the number of meetings between leucocytes and bacteria. The rates of phagocytosis calculated by the improved method were, therefore, corrected by multiplying by the viscosity of water as given in Landolt and Bornstein's tables before plotting. If the same correction had been applied to the original values of K they would have given a temperature coefficient less than the new values. The correction for viscosity decreases the value of Q_{10}, as shown in Table I, but does not appreciably affect the form of the curve.

It is conceivable that the number of collisions between bacteria and leucocytes might be so great that this would not be a limiting factor. In experiments on the phagocytosis of solid particles this certainly was not the case since the number of particles ingested in a given time was always a constant percentage of the number present.

The last column in Table I gives values for Q_{10} obtained when the time is measured from the end of the latent period instead of the beginning, the correction for viscosity also being made. This procedure gives a slightly lower value for Q_{10}.

We may take, then, for the temperature coefficient of phagocytosis, as nearly as it may be obtained from these experiments, the value 2.0.[3]

This figure is not thoroughly satisfactory, however, because the curves in Figs. 1 and 2 are complicated by at least two reactions, each with its own temperature coefficient, besides the phagocytic reaction proper.

The first of these reactions is represented by the latent period. Madsen and Watabiki calculated its temperature coefficient from the

[3] Ledingham, in studying the effect of temperature on phagocytosis of bacteria, concluded that it was due to the different rates of adsorption of opsonin by the bacteria. By previously incubating the bacteria with serum the accelerating effect of temperature could be nearly excluded. (Ledingham, J. C. G., *Proc. Roy. Soc. Biol., B.* 1907, lxxx, 188.)

length of the latent period by the formula of Arrhenius, and found $\mu = 15{,}000$ and 16,350 respectively in their paper.[2] The data fit the simpler formula of van't Hoff equally well and gives Q_{10} as 2.35 and 2.6, respectively.

A second complicating reaction is evidenced in Fig. 1 by the fact that at higher temperatures phagocytosis ceases; *i.e.*, the maximum is reached sooner than at lower temperatures. This indicates that some secondary reaction is occurring which results in injury to the cells or otherwise makes phagocytosis impossible.[4] The speed of this "lethal reaction," as we may call it, increases with the temperature. If the phagocytic reaction itself were accelerated by rise of temperature

TABLE I.

Temperature Coefficient of Phagocytosis.

	Uncorrected.	Corrected for viscosity.	Latent period deducted.
	Q_{10}	Q_{10}	Q_{10}
Fig. 2	2.7 ± 0.1	2.05	2.0
Fig. 3	2.5 ± 0.1	2.0	1.9

to the same degree as the lethal reaction, the maximum reached would be the same at all temperatures, for the more rapid phagocytosis at higher temperatures would just compensate for the shorter time available. Therefore, the fact that the maximum attained is higher at the higher temperatures, in spite of the shorter time, proves that the temperature coefficient of phagocytosis must be higher than the temperature coefficient of the lethal reaction.

In Fig. 4 are plotted graphs from which the Q_{10} of the lethal reaction can be approximated. Ordinates represent logarithms of the reciprocals of the time necessary for completion of the reaction, *i.e.*, time to reach the maximum; abscissæ represent temperature. These figures are, of course, highly inaccurate, probably particularly so at tem-

[4] The maximum cannot be due entirely to any mechanical factor such as the exhaustion of the available bacteria or the filling up of the cells or it would be the same in all. The lethal reaction which determines the maximum might be referred to the toxic action of bacterial extracts.

peratures below 15° where the reactions were continued for 24 and 48 hours; and it has seemed justifiable, in calculating the value of Q_{10}, to discount these figures on account of secondary changes which might occur in such prolonged experiments. If this is done, the values for the temperature coefficient are 1.7 and 1.3 respectively. This bears out the qualitative conclusion arrived at from mere inspection of the curves in Fig. 1 that the temperature coefficient of the reaction resulting in injury to the cells was lower than that for the process of phagocytosis itself.

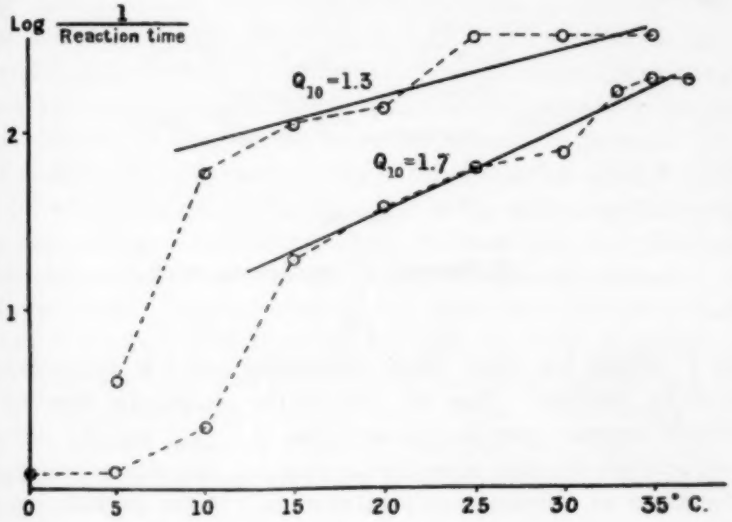
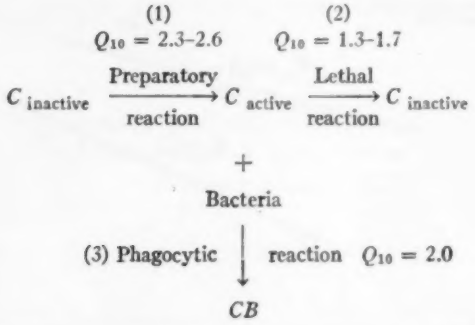


FIG. 4. Logarithms of the reciprocals of the times necessary to ingest the maximum number of bacteria (ordinates) plotted against temperature (abscissæ). Data taken from the same two experiments of Madsen and Watabiki as for the graphs of Figs. 2 (lower) and 3 (upper). Slope of the graph is proportional to the logarithm of the value of Q_{10} for that interval. Values calculated from the slopes of the solid lines are inserted. This is regarded as the temperature coefficient of the lethal reaction resulting in the death of the cell and cessation of phagocytosis. 3.75 and 2.5 have been added to the values for the upper and lower curves respectively for convenience in plotting.

Loeb (4) and Osterhout (5) have developed the conception of catenary reactions as applied to biological reactions, and the latter has recently discussed temperature coefficients from this point of view.

This general conception is obviously applicable to the experiments of Madsen and Watabiki, where three separate reactions can be clearly distinguished by mere inspection of their curves in Fig. 1. These reactions are: (1) a preparatory reaction represented by the latent period, during which it may be supposed that the leucocytes, originally inactive, are rendered active; (2) a lethal reaction which injures the leucocytes so that they are again inactive; and (3) the phagocytic reaction proper.

The relations between these three reactions may be diagrammed as follows:[5]



Here C stands for white blood corpuscles and CB for corpuscles containing bacteria. Now the rate of the phagocytic reaction (3) evidently depends upon the concentration of C_{active}; and the accuracy of our analysis depends upon the assumption that this concentration is the same at different temperatures during the periods selected as corresponding periods in the reactions. The concentration of C_{active} depends in turn upon the relative rates of the preparatory and lethal reactions. Could we know the rates and dynamics of both these reactions as well as their temperature coefficients we could calculate the variation of C_{active} with time by Osterhout's equations (6) for the calculation of the concentration of M in the series $A \rightarrow M \rightarrow B$. In this way corresponding stages could be ac-

[5] In this diagram no assumption is implied as to whether the effect of reactions (1) and (2) in "activating" and "inactivating" the cells is due to an action upon the cells themselves or upon the medium or bacteria. It merely states the fact that their phagocytic activity passes through a maximum during the experiment, due to two reactions.

curately selected. Unfortunately this is not possible. Moreover, the activity of the cells cannot be quite the same during the periods selected, since the higher temperature coefficient of the preparatory reaction compared to the lethal reaction would make the concentration of C_{active} pass through its maximum sooner at higher temperatures. An improvement might be made by taking as corresponding stages the times until the lethal reaction is one-half complete; *i.e.*, one-half the time necessary for cessation of phagocytosis instead of the time for the ingestion of one-half the total number of bacteria. The validity of this method depends upon the doubtful assumption that cessation of phagocytosis is caused entirely by the lethal reaction. It seems quite probable, however, particularly at the higher temperatures, that the filling up of the leucocytes with bacteria or the partial exhaustion of the free bacteria is another factor of importance. There is obviously a limit to the accuracy of interpretation which is possible. On the whole, the corresponding stages in the reaction probably would not differ so much from those which we have used that the *average* rates of ingestion in those periods would be seriously affected.

In conclusion, emphasis may be laid upon the central fact that the phagocytic curves of Madsen and Watabiki represent a complex of at least three reactions and consequently cannot be treated as a single monomolecular reaction without serious error. Osterhout's conception of catenary and (we may add) collateral reactions is not only applicable to the interpretation of these experiments, but obviously essential.

For comparison with the results of Madsen and Watabiki on the temperature coefficient of the phagocytosis of bacteria, it seemed of interest to determine the temperature coefficient of the phagocytosis of solid particles of carbon and quartz. For this purpose leucocytes obtained from a peritoneal exudate in the rat and particles of quartz or carbon of uniform sizes (2 to 4μ in diameter) were mixed in small glass-stoppered vials which were rotated slowly about their horizontal axes in water baths kept at the desired temperature. At frequent intervals small samples were removed and counts made of the number of particles not yet ingested. For the details of the procedure the reader is referred to a previous paper (2). The difficulty with the method is that both particles and leucocytes are likely to agglutinate

more or less during the experiment and that it is impossible to be sure whether the particles are actually inside the cells or merely stuck on the outside. Hence, if the particles stick on the outside more rapidly than they are ingested, it becomes impossible to measure the actual rate of ingestion or to determine the true temperature coefficient of ingestion. This is undoubtedly the explanation of the low temperature coefficient, 1.4, obtained by this method between 30° and 37°C.

The results of one experiment by the suspension method is shown in Fig. 5, where the percentage of particles *not* ingested is plotted as ordinates against time in minutes as abscissæ. Determinations were made at 37°, 27°, and 23°C. Inspection of these curves shows that the temperature coefficient is very low above 30°C. compared to that below 30°C. To obtain a quantitative comparison, the times, T, necessary for ingestion of 25, 50, and 75 per cent of the particles have been compared. Here, again, the rate of the reaction is conditioned in part by the viscosity of the medium, collisions taking place between cells and particles with proportionally less frequency in the more viscous medium at 23°C. The rate of the reaction, K, when corrected for viscosity is, then, the reciprocal of the time necessary for the ingestion of a given percentage of particles multiplied by the viscosity.

$$K = \frac{V}{T} \qquad (1)$$

The temperature coefficient, Q_{10}, is now calculated according to the formula

$$\text{Log } Q_{10} = \frac{\text{Log } K_1 - \text{Log } K_2}{t_1 - t_2} \times 10 \qquad (2)$$

where t is the temperature, as already explained. In this case the graph of log K against temperature is not a straight line as in the case of Madsen and Watabiki's results. In the experiment plotted in Fig. 5, Q_{10} is 1.26 between 23° and 27°C., and 5.6 between 27° and 37°C., when the figures for comparison are taken at the stage in the reaction when 50 per cent of the particles are ingested. The values obtained at other stages (25 and 75 per cent) in the reaction are given in Table II together with the results of seven other similar experiments. While the variations in the experimental figures are

large, the general tendency for Q_{10} to increase at lower temperatures is perfectly clean-cut.

The simplest interpretation of these results seems to be that at the lower temperatures the cells are actually too rigid to permit either the sticking on of the particles or the relatively greater changes in form which are necessary for ingestion. A slight increase in temperature now suffices to transform the protoplasm possibly from the *gel* to the

TABLE II.

Temperature Coefficient of Phagocytosis of Solid Particles.

Experiment No.	Particles		25 per cent ingested.	50 per cent ingested.	75 per cent ingested.	Remarks.
	Nature.	Size.				
		μ	Q_{10}	Q_{10}	Q_{10}	
1	Carbon.	4.7	1.1	1.3	1.0	Between 30°C. and 37°C. Grand average = 1.41 ± 0.06.
2	Quartz.	6.0	1.9	1.6		
3	"	2.4	1.7	1.1	1.1	
4	Carbon.	4.7		1.2	1.4	
5	"	4.7	2.0	2.3	1.6	
6	"	4.7	1.4	1.3		
7	Quartz.	4.6	1.0	1.8	1.0	
8	"	2.4	1.3	1.2	1.3	
1	Carbon.	4.7	5.9	5.6	14.0	Between 23°C. and 27°C. Grand average = 18 ± 6.
7	Quartz.	4.6	71.0	10.2	8.9	
9	Carbon.	4.7	10.7			

Comparisons were made at three different stages of the reaction; *i. e.*, when 25, 50, and 75 per cent of the particles had been ingested. In Experiments 1, 4, and 7, Q_{10} was measured from 27°C. to 37°C.

sol stage, thereby rapidly increasing the rate of phagocytosis; *i.e.*, Q_{10} is high. The low temperature coefficient above 30°C. might be taken to indicate that phagocytosis is merely a matter of surface tension changes. But this conclusion is not justified since many of the particles were merely stuck on the outside of the leucocytes singly or in clumps, making impossible any conclusion as to the temperature coefficient of the actual process of ingestion. The fact that no appreciable clumping occurs at lower temperatures shows that the

clumping is due to the increased stickiness or phagocytic activity of the cells.

In this connection the parallel fact is significant that the presence of solid particles has been found (2) to cause more rapid clumping of the leucocytes. Thus, at the optimum temperature for phagocytosis there is both an agglutination of cells due to particles and an agglutination of particles due to cells. The complete act of phagocytosis is

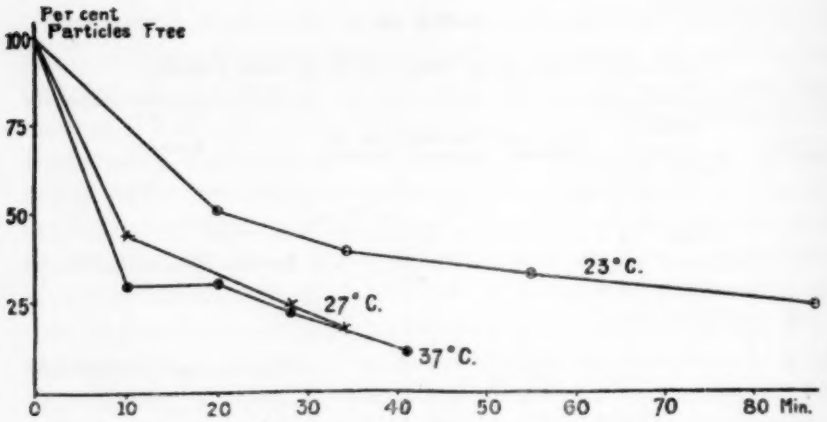


FIG. 5. Curves showing the number of particles of carbon still free (ordinates), *i.e.*, not ingested by leucocytes, as a function of time (abscissæ), at 23°, 27°, and 37°C. Inspection shows that the temperature coefficient is greater between 23° and 27°C. than between 27° and 37°C. See Table I for figures from this and other similar experiments.

evidently a complex of several reactions, and any figure which may be obtained for the temperature coefficient of the process must be interpreted from this point of view. The fact that Q_{10} is not constant must mean that new reactions become the limiting factors as the temperature changes. Below 30°C. the fluidity of the cell is the limiting factor. Above 30°C, it is the stickiness of the cells which would be expected to vary merely with the surface tension.

These results indicate then a marked change in the consistency of the protoplasm between 20° and 30°C. which did not appear in the

results of Madsen and Watabiki. Similar indications are found, however, in measurements of the temperature coefficient of the ameboid motion of leucocytes by Commandon (7) who took moving pictures of creeping leucocytes at 25°, 30°, and 35°C. and determined the rates of movement by subsequent measurements on the films. He gives the following figures:

Temperature.	Speed of leucocytes.	Q_{10}
°C.	*μ per minute*	
25	9.6	4.6
30	20.4	
30	20.4	1.5
35	25.2	

From these figures the Q_{10} has been calculated as described and was found to be 4.6 between 25° and 30°C., and 1.5 between 30° and 35°C.

Similar evidence of a rapid change in the temperature coefficient of ameboid movement below 30°C. was found in some preliminary experiments designed to measure the effect of temperature on phagocytosis by the "film method," previously described (2). In this method the leucocytes and particles are allowed to settle out between a slide and cover-slip in a thin film and the rate of phagocytosis is measured by counting the number of particles not yet ingested over equal areas. It is impossible to be sure, however, whether this rate is determined by the speed with which the leucocytes creep about from particle to particle or by the actual ease of ingestion. Demonstrations by this method, of the more rapid ingestion of carbon particles as compared with quartz, have shown that in this case at least the speed of the leucocytes is not the limiting factor. However that may be, the temperature coefficients of phagocytosis as measured by this method show the same marked increase at lower temperatures. This is shown in Table III.

TABLE III.

Temperature Coefficients of Phagocytosis by Film Method.

Experiment No.	Nature of particle.	Particles ingested.	27°–35°C.	20°–27°C.	10°–20°C.
		per cent	Q_{10}	Q_{10}	Q_{10}
10	Carbon.	25	4.2	7.9	6.8
		50	4.2	10.0	6.0
		75	7.1		
	Quartz.	25	1.7	20.0	7.6
		50	1.3		
		75	2.0		
11	Carbon.	25	3.2	9.3	
		50	4.0	8.7	
	Quartz.	25	2.0	25.0	
		50	1.6	18.6	
Averages.....			3.2± 0.4	14.2± 1.7	6.8

In each experiment a mixture of quartz particles 4.6 μ in diameter and carbon particles 4.7 μ was used. 0.3 cc. of this mixture in distilled water was added to 1 cc. of leucocyte suspension plus 0.4 cc. serum plus 0.2 cc. NaCl 2.25 per cent plus 0.2 cc. $\frac{M}{10}$ phosphate mixture of pH 7.5. Rates of phagocytosis, K, were taken equal to the reciprocal of the times, T, necessary for the ingestion of 25 per cent, 50 per cent, or 75 per cent of the particles. T was determined graphically. Q_{10} was calculated by equation (2). The dispersion of the average was calculated where the data were adequate.

SUMMARY.

1. The experiments of Madsen and Watabiki on the effect of temperature on the phagocytosis of bacteria are discussed and a new analysis of their curves is given, showing that the rate of phagocytosis is very nearly a logarithmic function of the temperature from 0° to 35°C.; *i.e.*, Q_{10} is constant over that range and is equal to 2.0.

2. New experiments are reported on the effect of temperature on the phagocytosis of quartz and carbon particles of uniform sizes, showing a marked increase in the temperature coefficient below 30°C.

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3. Osterhout, W. J. V., *Science*, 1918, xlviii, 172.
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THE PENETRATION OF CATIONS INTO LIVING CELLS.

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According to some investigators the living cell is impermeable to salts, while according to others it is permeable to anions but not to cations. The object of the present investigation is to throw some light on the problem by making direct determinations of the penetrating substances.

The literature of the permeability of protoplasm to salts has been summarized by Brooks[1] and will not be discussed here. Attention may, however, be called to the fact that satisfactory direct methods of study have been lacking. The importance of obtaining direct evidence of the penetration of substances into the protoplasm cannot be overestimated.

The demonstration of direct penetration has been very difficult because individual cells are usually so small that their contents cannot be analyzed. Meyer,[2] Hansen,[3] Wodehouse,[4] and Crozier,[5] have examined the cell contents of *Valonia* for evidence of penetration of salts from sea water. In this case the cell sap can be obtained without contamination and in sufficient quantities for examination.

By employing a large form of *Nitella* the writer was able to investigate the penetration of several cations from balanced and from unbalanced solutions. This species of *Nitella* is especially favorable because of the length of the (multinucleate) cells (5 inches is not unusual), and the amount of cell sap which can be expressed from a single cell.

[1] Brooks, S. C., *Bot. Gaz.*, 1917, lxiv, 230.
[2] Meyer, A., *Ber. deutsch. bot. Ges.*, 1891, ix, 79.
[3] Hansen, A., *Mitt. Zool. Stat. Neapel*, 1893, xi, 255.
[4] Wodehouse, R. P., *J. Biol. Chem.*, 1917, xxix, 453.
[5] Crozier, W. J., *J. Gen. Physiol.*, 1918–19, i, 581.

The cells were placed in the solutions[6] for various lengths of time. When they were taken out they were thoroughly rinsed in running tap water, dried with filter paper and pierced with a fine pointed capillary pipette, which collected the cell sap in a very satisfactory manner. This was tested by means of the spectroscope.

The normal cell contained Na, Ca, and Cl; for this reason Na and Ca were not used in making up the test solutions.

The cations used were Li, Cs, and Sr. In order to avoid plasmolysis hypotonic solutions were employed.

In order to make sure that the tests obtained were not due to small quantities of the solution adhering to the outside of the cell, cells were placed for half an hour or longer in each solution, taken out, washed in running water, and tested in the usual manner. The test was in all cases negative, showing that the method as ordinarily used excludes errors due to contamination.

When *Nitella* was placed in 0.05 M LiCl, penetration of Li could be demonstrated in 24 hours. At this concentration the salt was not toxic. The cells remained in good condition for 4 days (after which the experiment was discontinued). Other plants which had been placed in 0.025 M LiCl gave a faint test for Li in 48 hours and were found to be in excellent condition at the end of 13 days.

In a balanced solution of Li (containing 10 parts 0.04 M LiCl, 9 parts tap water, and 1 part sea water) the cell sap gave a good test in 48 hours and cells were found to be in excellent condition at the end of 16 days.

Other cells were placed in 0.05 M CsCl and gave a good test in 24 hours. These were under observation for 6 days, during which time they remained in good condition.

In a balanced solution containing 10 parts 0.05 M CsCl, 9 parts tap water, and 1 part sea water, the cell sap gave a good test for Cs in 3 days, and the cells remained in good condition for 4 days (after which the experiment was discontinued).

In $SrCl_2$ (0.075 M) *Nitella* remained alive for 20 days, and there was a slow penetration requiring 3 days or more to give a good test. The

[6] The solutions were approximately neutral. The temperature did not vary much from 19°C.

penetration of Sr from a balanced solution containing 10 parts $SrCl_2$ (0.075 M), 9 parts tap water, and 1 part sea water, was still slower.

These experiments demonstrate that Li, Cs, and Sr penetrate into the interior of the living cell and that this occurs more rapidly in an unbalanced than in a balanced solution. That they may penetrate in the form of ions is evident from the fact that in salts of these metals the electrical conductivity[7] of the living cell is such as to show that the cations Li, Cs, and Sr readily penetrate the protoplasm. Since this occurs at the very start of the experiment, before any appreciable injury has occurred, it is evident that the cell in its normal condition is permeable to these cations.

SUMMARY.

Direct tests of the cell sap of *Nitella* show that the protoplasm is normally permeable to Li, Cs, and Sr, and that penetration is more rapid in an unbalanced than in a balanced solution.

[7] These results were obtained by the method described by Osterhout (Osterhout, W. J. V., *J. Gen. Physiol.*, 1921–22, iv, 275).

THE ORIGIN OF THE ELECTRICAL CHARGES OF COLLOIDAL PARTICLES AND OF LIVING TISSUES.

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(Received for publication, November 19, 1921.)

I. Stability of Suspensions, Electrical Charges of Micellæ, and Donnan Equilibrium.

The stability of suspensions is, perhaps, the chief problem of a theory of colloidal behavior. Hardy has shown that this problem is linked with the problem of the origin of the electrical charges of the particles in suspension (which we will term micellæ, when they consist of aggregates of ions or molecules) inasmuch as the micellæ carrying a sufficiently large electrical charge will be forced by mutual electrostatic repulsion to stay in suspension. By his experiments on the migration of suspended particles of coagulated white of egg in an electrical field he proved that they have a positive charge in the presence of acid, a negative charge in the presence of alkali, and no charge at an intermediate point which he termed the isoelectric point of the particles. He was able to demonstrate that the stability of colloidal suspensions is a minimum at the isoelectric point.[1]

He and others found, moreover, that low concentrations of neutral salts diminish the stability of colloidal suspensions in the presence of acids or alkalies and that the efficient ion of the salt has the opposite sign of charge from the colloidal particle; since the precipitating efficiency of a salt increases rapidly with the valency of that ion of the salt which has the opposite sign of charge from the colloidal particle. It seemed natural to infer that the precipitation of colloidal

[1] Hardy, W. B., *Proc. Roy. Soc. London, Series B*, 1899–1900, lxvi, 110; *J. Physiol.*, 1903, xxix, p. xxvi. Wood, T. B., and Hardy, W. B., *Proc. Roy. Soc. London*, 1909, lxxxi, 38.

suspensions by low concentrations of a salt was caused by an annihilation of the charge of the colloidal particle. The problem of the stability of the colloidal suspension then developed into the problem of accounting for this peculiar behavior of the electrical charges of colloidal particles.

Hardy's original idea was that the H ions of the acid or OH ions of the alkali were adsorbed by the colloidal particle in preference to the other ions on account of their greater rapidity of migration; and this idea was also accepted by Perrin in his experiments on electrical endosmose, where it was necessary to account for the fact that certain membranes become positively charged in the presence of acid and negatively in the presence of alkali.[2] Those who accept this adsorption hypothesis explain the fact that the electrical charges of the particles are apparently diminished or destroyed by the addition of a salt on the assumption of a preferential adsorption of one of the ions of the salt; yet such an assumption is incompatible with the purely stoichiometrical behavior of proteins. It is also difficult to account for the fact that the addition of little acid increases while the addition of more acid depresses the electrical charge of micellæ on the basis of the adsorption hypothesis.

A second possibility was pointed out by the writer in 1904; namely, that Hardy's migration experiments might be explained in the case of proteins by the fact that proteins are amphoteric electrolytes which, in the presence of alkali, dissociate electrolytically by giving rise to a protein anion and, in the presence of acid, by giving rise to a protein cation while at the isoelectric point no protein ion would be formed.[3] While this idea is correct if applied to the migration of isolated protein ions in the electrical field, it cannot explain why the addition of a salt in low concentration should diminish the charge of aggregates of molecules and ions, the micellæ, except by assuming that in this case the electrolytic dissociation of the protein salts should be repressed. The concentration of salts required for the precipitation of colloidal suspensions is, however, much too small to make such a suggestion acceptable.

[2] Perrin, J., *J. chim. physique*, 1904, ii, 601; 1905, iii, 50.
[3] Loeb, J., *Univ. California Pub., Physiol.*, 1903–04, i, 149.

In 1916 J. A. Wilson suggested that the electrical charges of micellæ were caused by the establishment of a Donnan equilibrium between the colloidal particle and the surrounding solution.[4] There were, however, no measurements of membrane potentials available at that time and this was probably the reason that his suggestion was not accepted.

We may consider a solution of a protein inside a collodion bag surrounded by a watery solution (free from protein) as a model of a micella. The measurements of the P. D. with the aid of a Compton electrometer with saturated calomel electrodes show that the electrical charges of this model vary in the same way as the charges possessed by colloidal particles in general (*e.g.* coagulated egg albumin) in suspension; namely, (1) The electrical charge of the micella model is zero at the isoelectric point of the protein. (2) The charge of the model is positive on the acid side and negative on the alkali side of the isoelectric point, and increases with the addition of little acid and diminishes with the addition of more acid to isoelectric particles. (3) The charge of the model is depressed by the addition of low concentrations of neutral salts and the depressing action of the salt increases rapidly with the valency of that ion of the neutral salt which has the opposite sign of charge to that of the micella.[5]

If these charges are due to the Donnan equilibrium it must be possible to prove that the concentration of the crystalloidal ions inside the micella (or its model) is different from their concentration in the surrounding liquid and that this difference in the concentration of crystalloidal ions on the opposite sides of the membrane is able to account quantitatively for the observed P. D. The difference in the concentration of crystalloidal ions in the two phases (micella and surrounding water) is due to the fact that the protein ion cannot diffuse into the watery solution. When a solution of a protein-acid salt is inside a collodion membrane, the diffusion of the protein ion is prevented by the membrane and when the protein ion forms part of a gel the diffusion of the protein ions is prevented by the forces of

[4] Wilson, J. A., *J. Am. Chem. Soc.*, 1916, xxxviii, 1982.
[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667.

cohesion between the protein ions constituting the gel, while both the collodion membrane and the solid gel are permeable for crystalloidal ions. We have already shown that when we separate a solution of gelatin chloride from water by a collodion membrane, at equilibrium the concentration of chlorine ions inside the gelatin solution is greater than the concentration of chlorine ions in the outside solution, and the concentration of the hydrogen ions inside the gelatin solution is smaller than outside, as Donnan's theory demands.[5] The concentration of the Cl ions was determined by titration and it was shown that the P.D. calculated with the aid of Nernst's formula from the difference of concentration of chlorine ions inside and outside, was within the limits of accuracy of the measurements, identical with the P.D. directly observed between the gelatin chloride solution and the outside solution with the aid of a Compton electrometer with saturated calomel electrodes. In other words, it was found that

$$\text{P.D. observed} = 58 \log \frac{C_{\text{Cl outside}}}{C_{\text{Cl inside}}} \text{ millivolts}$$

or, since $\log C_{\text{Cl outside}} = \text{pCl outside}$, and $\log C_{\text{Cl inside}} = \text{pCl inside}$,

$$\text{P.D. observed} = 58 \text{ . (pCl outside minus pCl inside) millivolts}$$

If Donnan's theory accounts quantitatively for the observed P. D., the value 58 (pH inside minus pH outside) should also agree quantitatively with the observed P.D.

The values of pH inside and pH outside were determined in our experiments with the potentiometer (*i.e.* the hydrogen electrode), and the value 58 (pH inside minus pH outside) was therefore also an observed value. In order to avoid confusion of terms we call the P.D. observed with the Compton electrometer the *observed* P.D., since this gives us the empirical charge found in the micella or its model, free from any theory. On the other hand, we will call the value 58 (pH inside minus pH outside) the *calculated* P.D. since we could find this value by calculation from Nernst's formula if we determined the hydrogen ion concentration of the inside and outside solutions by titration instead of by the potentiometer.

The determinations of the value pH inside minus pH outside give the following results already published in a preceding paper:[5] (1)

At the isoelectric point of gelatin the value of pH inside minus pH outside is zero. (2) When the collodion bag contains a solution of gelatin-acid salt, the value pH inside minus pH outside is positive. (3) The value pH inside minus pH outside increases at first when acid is added to isoelectric gelatin with the increase in acid, but soon reaches a maximum and diminishes again upon the addition of further acid. It is shown that the P.D. observed with the Compton electrometer between the solution and the water varies in exactly the same way. (4) The addition of a neutral salt to a solution of gelatin chloride at the pH where the observed P.D. is about a maximum diminishes the value of pH inside minus pH outside in the same way as it diminishes the observed P.D. (5) The main fact was that the value 58 (pH inside minus pH outside) agreed quantitatively with the observed P.D.

These facts show that the P.D. between a gelatin chloride solution and a watery solution (separated by a collodion membrane) is caused exclusively by a difference in the concentration of diffusible ions inside and outside the gelatin solution. If there were a second source for the P.D., the P.D. obtained from the value 58 (pH inside minus pH outside) would be always smaller than the P.D. observed with the aid of a Compton electrometer. The reader will therefore see that the quantitative agreement between the values of 58 (pH inside minus pH outside) with the observed P.D. between the gelatin chloride solution and the outside solution is the essential proof that only the Donnan equilibrium is responsible for the difference of potential between the gelatin chloride solution and an outside watery solution.

This paper intends to fill out several gaps left in the preceding publication. Thus it was not proven that on the basis of the Donnan equilibrium the gelatin must have a negative charge on the alkali side of the isoelectric point. When we put a solution of Na gelatinate into a collodion bag and dip the bag into water, the Donnan equilibrium demands that NaOH be expelled from the solution of Na gelatinate through the collodion membrane into the outside solution, and that when equilibrium is established between the solutions of Na gelatinate and water the concentration of NaOH must be greater in the outside watery solution than in the solution of Na

gelatinate (inside solution). As a consequence the pH in the outside solution should be higher than in the inside solution, and the value pH inside minus pH outside should become negative when the inside solution is Na gelatinate. This is the reason why powdered particles of Na gelatinate must assume a negative charge. In the case of a gelatin chloride solution the value pH inside minus pH outside is positive and this explains why powdered particles of gelatin chloride are positively charged.

We will now show that when we separate a solution of Na gelatinate from a watery solution by a collodion membrane and allow both solutions to reach or approach equilibrium, the value pH inside minus pH outside actually becomes negative.

II. The Electrical Charge of Na Gelatinate.

It is necessary to prevent the CO_2 of the air from diffusing into the solutions of Na gelatinate, and therefore the outside solution was put into stoppered bottles connected with the outside air by glass tubes filled with soda lime. The pH measurements were less certain than in the experiments with acid on account of the CO_2 error. There may be other experimental shortcomings, *e.g.*, the slowness of the establishment of the Donnan equilibrium between inside and outside solutions near the isoelectric point.

Collodion bags of a volume of about 50 cc. were filled with solutions of Na gelatinate containing 1 gm. of originally isoelectric gelatin and varying amounts of 0.1 N NaOH in 100 cc. solution. The collodion bags were dipped into flasks containing 500 cc. of aqueous solutions of NaOH of various concentrations and free from gelatin. The flasks were sealed, communicating with the air only through tubes filled with soda lime, as stated. The collodion bags containing the gelatin were closed by a rubber stopper perforated by a glass tube which served as a manometer. The experiment lasted 6 hours at a temperature of 24°C. The results of the experiments are given in Table I. The upper horizontal row gives the number of cc. of 0.1 N NaOH originally in 100 cc. of the gelatin solution; the second row gives the original concentration of NaOH in the outside aqueous solution free from gelatin; the third row gives the osmotic pressure in millimeters H_2O after 6 hours. The next row gives the pH inside

TABLE I.

1 Per Cent Na Gelatinate.

Cc. 0.1 N NaOH added to 1 gm. gelatin in 100 cc.	0	1	2	3	4	5	6	8	10	12.5	15	20
Concentration of NaOH of outside solution	0	0	0	N/25,600	N/12,800	N/6,400	N/3,200	N/1,600	N/800	N/400	N/200	N/100
Osmotic pressure, millimeters	26	164	265	353	375	385	366	335	340	265	192	150
pH inside	5.02	5.40	5.76	6.64	7.15	9.02	9.68	10.16	10.45	—	11.30	11.58
pH outside	5.60	5.82	5.92	6.37	7.70	9.50	9.96	10.60	10.85	—	11.46	11.70
pH inside minus pH outside	−0.58	−0.42	−0.16	+0.27	−0.55	−0.48	−0.28	−0.44	−0.40	—	−0.16	−0.12
P.D. calculated, millivolts	−34.0	−24.5	−9.4	+15.8	−32.0	−28.0	−16.5	−25.7	−23.4	—	−9.4	−7.0
P.D. observed, millivolts	−3.5	−19.5	−18.0	−37.5	−37.5	−36.0	−30.0	−22.0	−19.5	—	−10.0	−7.0

and the following row the pH outside after the experiment was finished (*i.e.* after 20 hours), and the sixth row gives the difference pH inside minus pH outside. The reader will notice that this difference is always negative with one exception which is obviously an error. The last two rows give the calculated P.D. (*i.e.* the value 58 (pH inside minus pH outside)), and the observed P.D. Observed P.D. means the millivolts between the solution of Na gelatinate and watery solution measured directly in the Compton electrometer, as described in preceding papers.

It is obvious that there is no quantitative agreement between the observed and calculated P.D. near the isoelectric point, probably on account of the CO_2 error. As soon as the pH is above 7.0 the agreement between observed and calculated P.D. becomes better so that we are entitled to say that the difference of potential between a Na gelatinate solution and an outside solution is due to the Donnan equilibrium which forces the expulsion of NaOH from the inside into the outside solution. As a consequence the pH inside becomes lower than the pH outside.

III. Valency Effect on the P.D.

It had been shown in a preceding paper that the charge of a solution of gelatin sulfate is lower than a charge of a solution of gelatin chloride or gelatin phosphate or any other gelatin-acid salt with a monovalent anion of the same pH and the same concentration of originally isoelectric gelatin.[5] It should, however, be pointed out that on the basis of the theory the ratio of the charges in the two cases should be exactly as 3:2. It is needless to say that if we can prove that this postulate is fulfilled the probability that the charges of micellæ are due to the Donnan equilibrium becomes very strong.

The equilibrium equation which is of the second degree when the anion is monovalent, *e.g.* Cl, in the case of gelatin chloride, becomes of the third degree when the anion is bivalent, *e.g.* SO_4, in the case of gelatin sulfate. Let x be the concentration of hydrogen ions and Cl ions of the outside, y that of the H and Cl ions of the free HCl inside the gelatin chloride solution, and z the concentration of the Cl in combination with gelatin. Then the equilibrium equation is,

$$x^2 = y\,(y+z)$$
$$x = \sqrt{y\,(y+z)}$$

Substituting this term $\sqrt{y\,(y+z)}$ for x in $\frac{x}{y}$ we get

$$\frac{x}{y} = \frac{\sqrt{y\,(y+z)}}{y} = \sqrt{1+\frac{z}{y}}$$

The P.D. is $58 \log \sqrt{1+\frac{z}{y}} = \frac{58}{2} \log \left(1+\frac{z}{y}\right)$.

In the same way we can arrive at the term for $\frac{x}{y}$ in the case of gelatin sulfate solution. If we call x the concentration of hydrogen ions in the outside solution, y that of the hydrogen ion concentration in the inside solution; then $\frac{x}{2}$ is the concentration of the SO_4 ions in the outside and $\frac{y}{2}$ the concentration of the SO_4 ions of the free H_2SO_4 in the inside (gelatin) solution. The concentration of SO_4 ions in combination with gelatin becomes $\frac{z}{2}$. Then the equilibrium equation is as follows:

$$\frac{x^3}{2} = \frac{y^2\,(y+z)}{2};\quad x = [y^2\,(y+z)]^{\frac{1}{3}}$$

The value which interests us is $\frac{x}{y}$, *i.e.* the ratio of the hydrogen ion concentration outside over that inside.

Substituting $[y^2\,(y+z)]^{\frac{1}{3}}$ for x in $\frac{x}{y}$ we get

$$\frac{x}{y} = \sqrt[3]{\frac{y^2\,(y+z)}{y^3}} = \sqrt[3]{\frac{y+z}{y}}$$

The P. D. is therefore in the case of gelatin sulfate

$$\text{P.D.} = \frac{58}{3} \log \left(1+\frac{z}{y}\right) \text{millivolts}$$

while in the case of gelatin chloride it is

$$\text{P.D.} = \frac{58}{2} \log \left(1+\frac{x}{y}\right) \text{millivolts}$$

Hence the P.D. of gelatin sulfate solution should be two-thirds of the P. D. of a gelatin chloride solution of the same pH and the same concentration of originally isoelectric gelatin.

1 gm. of isoelectric gelatin was dissolved in 100 cc. of water containing in one case 5 cc. of 0.1 N HCl, in the other, 5 cc. of 0.1 N H_2SO_4. One collodion bag with a volume of 50 cc. was filled with the gelatin chloride solution and this bag was dipped into a beaker containing 350 cc. N/1000 HCl. A second collodion bag was filled with the gelatin sulfate solution and this bag was dipped into 350 cc. N/1000 H_2SO_4. The solutions were kept for 24 hours at 24°C. and the pH inside, and pH outside were measured. The pH of the two gelatin solutions was identical, namely 3.64, but the value pH inside minus pH outside was for the gelatin chloride solution 0.49 and for the gelatin sulfate solution 0.31, which is as near 3:2 as the accuracy of the measurements permits. A confirmation of this result can be found in the experiments published in a preceding paper where this relation had not yet been recognized.[5] Thus it was found that for gelatin phosphate of pH 3.98 the value of pH inside minus pH outside was 0.58, while for gelatin sulfate of pH 3.98 the value of pH inside minus pH outside was 0.38, which is again the ratio of 3:2. For pH 4.31 the value pH inside minus pH outside was 0.53 for gelatin chloride while it was for pH 4.34, 0.35 for gelatin sulfate, which is again as 3:2. The P.D. observed directly with the Compton electrometer agreed quantitatively with the value 58 (pH inside minus pH outside).

Quantitative results, such as these, leave little doubt that the P. D. between solutions of gelatin-acid salts and outside watery solutions when separated by a collodion membrane are determined entirely by the Donnan equilibrium; and that there can be no other source of the charge of this micella model.

IV. The P. D. *of Solutions of Crystalline Egg Albumin.*

The experiments published thus far had all been done on gelatin. It was of importance to make sure whether or not these results can be confirmed with crystalline egg albumin. This was found to be the case, and the experiments on the membrane potentials of the

solutions of the chloride of crystalline egg albumin showed a perfect quantitative agreement with the theory.

Collodion bags of about 50 cc. volume were filled with a solution of 1 per cent crystalline egg albumin containing varying amounts of 0.1 N HCl, and the bags were put, as usual, into beakers containing 350 cc. of HCl solutions of different concentration but free from albumin. The first two horizontal rows of Table II give the amount of 0.1 N HCl in each solution. The experiments were carried out at a temperature of 24°C. and after 22 hours the osmotic pressure, P. D., and pH of inside (albumin) solution and pH of the outside solution were measured, the P. D. with the Compton electrometer and the pH with the hydrogen electrode. The albumin used was not isoelectric, but, since it had been prepared after Sörensen's method, it was probably partly ammonium albuminate, with a pH of near 6.0. The table shows that the observed P. D. agree with the value 58 (pH inside minus pH outside), *i.e.* the calculated P. D. (especially on the acid side of the isoelectric point); that the P. D. is a minimum near pH 4.7 of the albumin (*i.e.* near its isoelectric point which is at pH 4.8), and that the albumin is positively charged on the acid and negatively charged on the alkaline side of the isoelectric point. This is again in harmony with what we should expect on the basis of the Donnan equilibrium.

The next problem was to determine the influence of the addition of a neutral salt to a solution of the chloride of crystalline egg albumin. A 1 per cent solution of crystalline egg albumin containing 7 cc. of 0.1 N HCl in 100 cc. was made up in various concentrations of NaCl. The collodion bags containing these albumin chloride-NaCl mixtures were dipped into beakers containing 350 cc. of the same concentration of NaCl as that of the albumin solution, and all made up in N/1000 HCl. The experiment was carried out at 24°C. and the measurements were made after 22 hours.

Table III gives the results which show again a good agreement between the observed P. D. and the value 58 (pH inside minus pH outside), our so called calculated P.D.

We may, therefore, conclude that the P.D. of both gelatin solutions and solutions of crystalline egg albumin separated by a collodion membrane from a watery solution free from protein is accounted for

TABLE II.

1 Per Cent Albumin Chloride.

Cc. 0.1 N HCl added to 1 gm. albumin in 100 cc.	0	1	2	3	4	5	6	7	8	10	15	20	30	40
Cc. 0.1 N HCl in 350 cc. outside solution	0	0.1	0.3	0.5	1	1.5	2.1	2.8	4	7.1	16.4	32	60	80
Osmotic pressure, millimeters	155	100	52	114	178	205	214	219	218	180	136	100	81	74
pH inside	5.80	5.40	4.70	4.30	4.00	3.75	3.64	3.42	3.24	3.00	2.53	2.20	1.89	1.73
pH outside	6.14	5.64	4.67	4.06	3.65	3.38	3.22	3.07	2.91	2.71	2.37	2.10	1.82	1.70
pH inside minus pH outside	−0.34	−0.24	+0.03	+0.24	+0.35	+0.37	+0.42	+0.35	+0.33	+0.29	+0.16	+0.10	+0.07	+0.03
P.D. calculated, millivolts	−20.0	−14.0	+2.0	+14.0	+20.6	+22.4	+25.5	+21.0	+20.0	+17.5	+9.4	+6.0	+4.0	+2.0
P.D. observed, millivolts	−24.0	−16.0	+3.0	+11.5	+19.0	+19.5	+20.5	+19.5	+18.5	+16.0	+11.0	+10.0	+4.0	+3.5

TABLE III.

Influence of Salt on P.D. *of Albumin Chloride Solution.*

	Concentration of NaCl.									
	0	M/2,048	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
Osmotic pressure, millimeters.....	210	181	156	131	107	87	73	61	54	45
pH inside.....	3.35	3.32	3.32	3.27	3.25	3.20	3.19	3.22	3.21	3.22
pH outside.....	3.04	3.04	3.07	3.10	3.11	3.13	3.14	3.18	3.21	3.23
pH inside minus pH outside,.....	0.31	0.28	0.25	0.17	0.14	0.07	0.05	0.04	0.00	−0.01
P.D. calculated, millivolts.....	+18.0	+16.2	+14.5	+10.0	+8.0	+4.1	+2.9	+2.3	0	−0.5
P.D. observed, millivolts.....	+18.5	+15.5	+13.5	+10.0	+7.5	+5.0	+3.0	+1.5	+1.0	+0.5

completely by the Donnan equilibrium. There can be no other source for the electrical charge of this model of a protein micella except that due to the membrane equilibrium.

V. The Electrical Charges of Suspended Particles of Powdered Gelatin.

It is possible to show that the electrical charges of the powdered particles of gelatin suspended in a watery solution are determined by the fact that acid is forced from the suspended particles into the watery solution when the particles consist of gelatin chloride, and that alkali is forced from the particles into the solution when they consist of Na gelatinate.

Measurements of the P.D. between solid gels of gelatin and the surrounding solution suffer from inaccuracies (especially near the isoelectric point) which we have not been able to eliminate, so that we must be satisfied with only an approximate confirmation of the theory. In order to prove or to make it probable that the P.D. is due to the Donnan equilibrium we must be able to show that there exists a difference of the value of pH inside and pH outside the gel when the suspended particles of gelatin chloride or Na gelatinate are in equilibrium with the watery solution.

1 gm. of powdered gelatin of grain size between mesh 30 and 60, and rendered isoelectric was put into each of a series of closed flasks containing 350 cc. of distilled water with varying quantities of 0.1 N HCl or NaOH per 100 cc. (see Table IV). The temperature was 20°C. After 4 hours the powdered gelatin was separated from its liquid by filtration, the gelatin was melted and the pH of the melted gelatin and of the outside solution (filtrate) were measured. The gelatin was then solidified and the P. D. between the solid gelatin and the filtrate (outside solution) determined, as will be described a little later. The results of the experiments are given in Table IV. The first row gives the number of cc. of 0.1 N HCl or NaOH contained originally in 100 cc. outside solution. The next row gives the relative volume of the solid mass of gelatin, *i.e.* the degree of swelling. The rest of the table needs no explanation. It is obvious that pH inside minus pH outside is positive as long as the pH of the gelatin is on the acid side of the isoelectric point, while it is negative when the gelatin is on the alkaline side of the isoelectric point. The turning point is

TABLE IV.

Suspensions of Powdered Gelatin.

	HCl.				NaOH.						
Cc. 0.1 N HCl or NaOH in 100 cc. solution	1.0	0.5	0.2	0.1	0	0.1	0.2	0.5	1.0	2.0	4.0
Volume of gelatin, millimeters	28	20	18	16	17	18	28	37	40	47	48
pH of melted gelatin (inside)	4.44	4.56	4.79	4.85	4.89	4.98	5.06	5.50	6.74	9.54	10.15
pH of supernatant liquid (outside)	3.35	3.55	3.92	4.24	4.97	5.96	6.24	6.46	7.30	10.56	11.08
pH inside minus pH outside	+1.09	+1.01	+0.87	+0.61	−0.08	−0.98	−1.18	−0.96	−0.56	−1.02	−0.93
P.D. calculated, millivolts	+63.0	+58.6	+51.0	+36.0	−4.5	−57.0	−68.0	−56.0	−33.0	−59.0	−48.0
P.D. observed, millivolts	+56.0	+55.5	+36.5	+15.0	−17.5	−59.0	−61.0	−70.0	−66.0	−46.0	−36.0

approximately at the isoelectric point, but the measurements near the isoelectric point are obviously vitiated by experimental errors or by some other factor so that we cannot demonstrate more by the experiment than that the suspended particles of solid metal gelatinate have the opposite sign of charge from the gelatin chloride and that this difference is accompanied by a reversal of the sign of the value of pH inside minus pH outside, which is positive in the case of gelatin chloride and negative in the case of Na gelatinate. It may also be pointed out that the minimum of swelling (volume) coincides with the minimum of P.D.

While the experimental errors are rather great in the neighborhood of the isoelectric point and on the alkaline side, they are fortunately less annoying on the acid side when the hydrogen ion concentration is sufficiently large. In this case the agreement between the value pH inside minus pH outside and the P.D. observed is at least sufficient to show that the P.D. is caused by the Donnan equilibrium.

1 gm. of powdered isoelectric gelatin going through mesh 30 but not through mesh 60 was put into 350 cc. of water containing various quantities of HCl (see first horizontal row of Table V), and left in this solution for 24 hours at 20°C. The flasks were occasionally stirred. After 24 hours the volume of the particles was measured and they were put on a filter to allow the outside solution to drain off. The gelatin was then melted by heating to 45°C. and poured into glass cylinders which at their lower end had two glass side tubes attached. The mass was then allowed to solidify and the P. D. between gelatin and watery solution was ascertained. One of the two glass tubes dipped into a beaker containing the outside HCl solution (the filtrate) with which the gelatin had been in equilibrium, and the other dipped into a beaker containing a saturated solution of KCl. Each beaker was connected with one of the calomel electrodes (filled with saturated KCl) of a Compton electrometer. The last row in Table V gives the observed P.D. in millivolts.

The gelatin was then melted and its pH was determined potentiometrically. This is called pH inside in Table V. The pH of the outside solutions (filtrate) was also determined at 24°C.

While the agreement between the observed P.D. and the values of 58 (pH inside minus pH outside) is not as complete as in the experi-

TABLE V.

Suspensions of Powdered Gelatin.

Cc. 0.1 N HCl in 100 cc. H_2O	0.5	1.0	2.0	4.0	6.0	8.0	10.0	12.0	15.0	20.0	30.0	40.0
Volume of gelatin, millimeters	30	40	62	73	75	73	66	64	54	50	41	37
pH of melted gelatin (inside)	4.58	4.27	3.76	3.26	2.92	2.57	2.41	2.29	2.11	1.96	1.78	1.59
pH of supernatant liquid (outside)	3.89	3.45	3.04	2.65	2.44	2.27	2.16	2.07	1.95	1.82	1.65	1.49
pH inside minus pH outside	0.69	0.82	0.72	0.61	0.48	0.30	0.25	0.22	0.16	0.14	0.13	0.10
P.D. calculated, millivolts	+40.7	+48.4	+42.5	+36.0	+28.4	+17.7	+14.7	+13.0	+9.5	+8.3	+7.7	+5.9
P.D. observed, millivolts	+37.5	+39.0	+38.0	+29.5	+22.0	+17.7	+17.7	+18.2	+17.0	+10.7	+8.6	+5.4

ments with solutions inside collodion bags, it is at least sufficient to leave no doubt that this difference in pH inside and outside causes the P.D. In other words, there is no doubt that the P.D. between the powdered particles and the surrounding liquid with which they are in equilibrium is due to the Donnan equilibrium.

We have already shown in a preceding paper that the addition of a salt to a solution containing suspended particles of powdered gel of gelatin chloride diminishes the P.D. between the particles and surrounding liquid and that this diminution is due to a diminution of the value pH inside minus pH outside; *i.e.*, to the Donnan equilibrium.[5]

These facts then leave no doubt that the difference in the hydrogen ion concentration between micellæ of protein and the surrounding solution which the Donnan equilibrium demands is the only cause of the electrical charges of micellæ of proteins or of their models.

The experiments on the solution of casein chloride published by Robert F. Loeb and the writer in the preceding number of this Journal indicate that aside from the electrical charges osmotic forces may play a rôle in maintaining the stability of colloidal suspensions.[6] These forces are also a consequence of the Donnan equilibrium and hence vary in a similar way as the P.D. No other theory except the Donnan theory can account for this similarity.

VI. The Origin of the Electrical Charges of Living Cells and Tissues.

In his first paper on the theory of membrane equilibria Donnan suggested that the membrane potentials postulated by his theory might contribute towards an explanation of the action of nerves and even of electrical fish. In 1911 the writer suggested to Dr. Beutner that he investigate the P.D. between such organs as apples or leaves of the rubber plant and water, instead of the P.D. of muscles or nerves which had usually been used by physiologists for this purpose. In these experiments Dr. Beutner made the important observation that the P.D. between the surface of an apple or a leaf was a maximum when the bounding liquid was pure water, while the P. D. was depressed when a salt was added to the water the depressing effect on

[6] Loeb, J., and Loeb, R. F., *J. Gen. Physiol.*, 1921–22, iv, 187.

the P. D. increasing with the concentration of the salt.[7] MacDonald[8] had observed a similar phenomenon; namely, the increase in P.D. between nerve and surrounding salt solution with increasing dilution. Donnan's theory was not known to us and we were not able to give an explanation of the depressing effect of salt on the P.D.

We next searched for those substances in the cortex of an apple or leaf which might be responsible for these peculiar concentration effects on the P. D. When the P. D. between solid gels of gelatin and of coagulated egg albumin and water was investigated no potential differences were observed,[9] to the great surprise and disappointment of the writer who had hoped that the investigations of the P. D. might lead to an explanation of the antagonistic ion effects in which he was then interested. It is possible that the negative results with protein were due to the fact that the measurements were accidentally made near the isoelectric point. On the other hand, it was found that there exists a P.D. at the boundary of lipoids (lecithin dissolved in guaiacol) which is depressed by the addition of salts, and the more the higher the concentration of the salt.[9]

This analogy between lipoids and living cells gave us the impression that the proteins had no share in the potential differences observed between living tissues or living cells and watery solutions. The experiments recorded in this paper leave no doubt that this conclusion was wrong; any ion in a cell or on its surface which cannot diffuse into the surrounding watery solution (no matter whether the ion is a protein or a fatty acid or some complicated lipoid or a complicated carbohydrate or even a crystalloid) can or must give rise to a P.D. which is depressed when a diffusible salt is added to the surrounding watery solution.

The idea that lipoids are the substances responsible for the P.D. of tissues led Beutner to an extensive and most interesting investigation of the P.D. at the boundary of water-immiscible substances and water.[10] He found always a depressing effect of the addition of salt. Beutner

[7] Loeb, J., and Beutner, R., *Biochem. Z.*, 1912, xli, 1.

[8] MacDonald, J. S., *Proc. Roy. Soc.*, 1900, lxvii, 310.

[9] Loeb, J., and Beutner, R., *Biochem. Z.*, 1913, li, 288; 1914, lix, 195.

[10] Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920.

tried to explain this on the basis of differences in the electrolytic dissociation in the watery and the water-immiscible (oily) phase. Such an explanation cannot be applied to the experiments with protein solutions and yet these latter solutions also show the depressing effect of the addition of salt on the P.D. in a most striking way. In this latter case the depressing effect of the salt on the P.D. is due to the Donnan equilibrium and there is no reason why the theory of membrane equilibria should not apply to the P.D. between oily and watery phases since this theory only demands that one ion of the oily phase should be prevented from migrating into the watery phase. Any lipoid ion would fulfill this postulate of the theory. The peculiarities of electrolytic dissociation found by Beutner in non-aqueous solutions must, however, influence the Donnan equilibrium in a secondary way since this equilibrium depends on ionization.

SUMMARY AND CONCLUSIONS.

1. When a solution of a salt of gelatin or crystalline egg albumin is separated by a collodion membrane from a watery solution (free from protein) a potential difference is set up across the membrane in which the protein is positively charged in the case of protein-acid salts and in which the protein is negatively charged in the case of metal proteinates. The turning point is the isoelectric point of the protein.

2. Measurements of the pH of the (inside) protein solution and of the outside watery solution show that when equilibrium is established the value pH inside minus pH outside is positive in the case of protein-acid salts and negative in the case of metal proteinates. This is to be expected when the P.D. is caused by the establishment of a Donnan equilibrium, since in that case the pH should be lower outside than inside in the case of a protein-acid salt and should be higher outside than inside in the case of a metal proteinate.

3. At the isoelectric point where the electrical charge is zero the value of pH inside minus pH outside becomes also zero.

4. It is shown that a P. D. is established between suspended particles of powdered gelatin and the surrounding watery solution and that the sign of charge of the particles is positive when they contain gelatin-acid salts, while it is negative when the powdered particles contain metal gelatinate. At the isoelectric point the charge is zero.

5. Measurements of the pH inside the powdered particles and of the pH in the outside watery solution show that when equilibrium is established the value pH inside minus pH outside is positive when the powdered particles contain a gelatin-acid salt, while the value pH inside minus pH outside is negative when the powdered particles contain Na gelatinate. At the isoelectric point the value pH inside minus pH outside is zero.

6. The addition of neutral salts depresses the electrical charge of the powdered particles of protein-acid salts. It is shown that the addition of salts to a suspension of powdered particles of gelatin chloride also diminishes the value of pH inside minus pH outside.

7. The agreement between the values 58 (pH inside minus pH outside) and the P. D. observed by the Compton electrometer is not only qualitative but quantitative. This proves that the difference in the concentration of acid (or alkali, as the case may be) in the two phases is the only cause for the observed P.D.

8. The Donnan theory demands that the P.D. of a gelatin chloride solution should be $1\frac{1}{2}$ times as great as the P.D. of a gelatin sulfate solution of the same pH and the same concentration (1 per cent) of originally isoelectric gelatin. This is found to be correct and it is also shown that the values of pH inside minus pH outside for the two solutions possess the ratio of 3:2.

9. All these measurements prove that the electrical charges of suspended particles of protein are determined exclusively by the Donnan equilibrium.